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TARGET VALIDATION OF UK-101 AND FUNCTIONAL STUDIES OF β1i

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TARGET VALIDATION OF UK-101 AND FUNCTIONAL STUDIES OF β1i

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By

Marie V. Wehenkel

Lexington, Kentucky

Director: Dr. Kyung Bo Kim, Professor of Pharmaceutical Sciences

Lexington, Kentucky

2011

ABSTRACT OF DISSERTATION

TARGET VALIDATION OF UK-101 AND FUNCTIONAL STUDIES OF β1i

β1i is a major catalytic subunit of the immunoproteasome, an alternative form of the constitutive proteasome, and its upregulation has been demonstrated in a variety of disease states including cancer. Our lab has developed a small molecule inhibitor of β1i, dubbed UK-101. While UK-101 causes apoptosis in cancer cell lines, it was not clear whether this apoptotic effect was directly mediated by its irreversible inhibition of β1i. Since off-target effects are major roadblocks for the development of new and effective pharmaceuticals, target validation studies in this system would assist in the further progression of β1i inhibitors towards preclinical trials. Our hypothesis was that the expression and catalytic activity of β1i is important for the growth and proliferation of the PC-3 prostate cancer cell line, therefore the apoptotic effect seen upon treatment of PC-3 cells with UK-101 was due solely to its covalent inhibition of β1i.

To test this hypothesis, a number of complementary approaches were used. The expression of β1i in PC-3 cells was increased by the treatment of these cells with interferon-gamma or tumor necrosis factor-alpha, natural inducers of the immunoproteasome. The expression of β1i in PC-3 cells was decreased using small interfering RNA or short hairpin RNA, in a transient or stable manner, respectively. All of these cells were then treated with UK-101. The efficacy of UK-101 decreased in the interferon-gamma treated cells but did not change in any other the other cell lines, suggesting that UK-101 was not specific for β1i. This was confirmed using a molecular probe of the proteasome and demonstrated that UK-101 bound to other proteasome catalytic subunits.

Additional experiments were performed to determine the effect of β1i on the proliferation of PC-3 cells. Simply removing the β1i using small interfering RNA reduces the viability of these cells. Other studies demonstrated that a mutation of β1i which inhibited its catalytic activity reduced the viability of cells when compared to those containing the wild type protein. Overall, our data indicate that β1i is a potential therapeutic target in prostate cancer. Further medicinal chemistry efforts will be required develop UK-101 into a truly selective proteasome inhibitor.

KEYWORDS: Immunoproteasome, Target Validation, β1i, UK-101, proteasome inhibitor *(Include exactly 5 keywords or phrases)*

Marie Wehenkel
Student's Signature

December 15, 2011
Date

TARGET VALIDATION OF UK-101 AND FUNCTIONAL STUDIES OF β1i

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December 15, 2011

To my parents, whom, when faced with a child who never stopped asking, “Why?” always encouraged her seek out the answers. Your constant support and encouragement made the difference. I love you both.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1: Introduction

A: Intracellular proteolysis and the ubiquitin-proteasome pathway

Scientists spent much of the 1900s studying the flow of genetic information into proteins, building up the evidence for what is now known as the "central dogma of molecular biology." Prior to 1950, few scientists were concerned with the destruction of proteins, as most believed intracellular proteins to be extremely stable and resilient. The lysosome, discovered in the 1950s, was thought to provide cells with the necessary destructive capacity. Over the next two decades it became increasingly difficult to reconcile the mechanistic understanding of lysosomal degradation with the turnover of intracellular proteins, especially those that were short-lived or abnormal. It was also found that much of this intracellular protein turnover required adenosine triphosphate (ATP), in direct conflict with the mechanism of action of lysosomes.

Work done in the early 1980s by Aaron Ciechanover, under the direction of Avram Hershko and his collaborator Irwin Rose, led to "the discovery of ubiquitin-mediated protein degradation" for which the trio received the 2004 Nobel Prize in Chemistry. [1] Their work explained the ATP-dependency of intracellular protein degradation by demonstrating that proteins are degraded more effectively when tagged with a protein called ubiquitin. A three-enzyme cascade was discovered which adds ubiquitins to proteins in a specific and ATP-dependent manner (Figure 1.1). The ubiquitin-activating enzyme class (E1) uses ATP to charge and transfer ubiquitin to a second enzyme class, the ubiquitin carrier proteins (E2). These E2 enzymes associate with the third class of enzymes, the ubiquitin protein ligases (E3), which can add ubiquitin molecules to proteins in a substrate-specific manner. Likewise, later work discovered an additional class of enzymes, called deubiquitinases (DUBs), which can remove ubiquitin moieties from proteins and thus further regulate the pathway.

The next critical step was to determine the protease responsible for the eventual degradation of these ubiqutinated proteins. By the end of the 1980s, many of the leaders in this young field of protein degradation believed the proteasome might be the ATP-dependent protease which recognizes and degrades ubiquitinated proteins. This complex was first described by S. Wilk and M. Orlowski in the early 1980s and was then known as the multicatalytic protease complex (Figure 1.1). [2, 3] The multicatalytic protease complex, or proteasome, was a ~700kDa complex found in electron microscopy studies to be a hollow, cylindrical structure composed of four

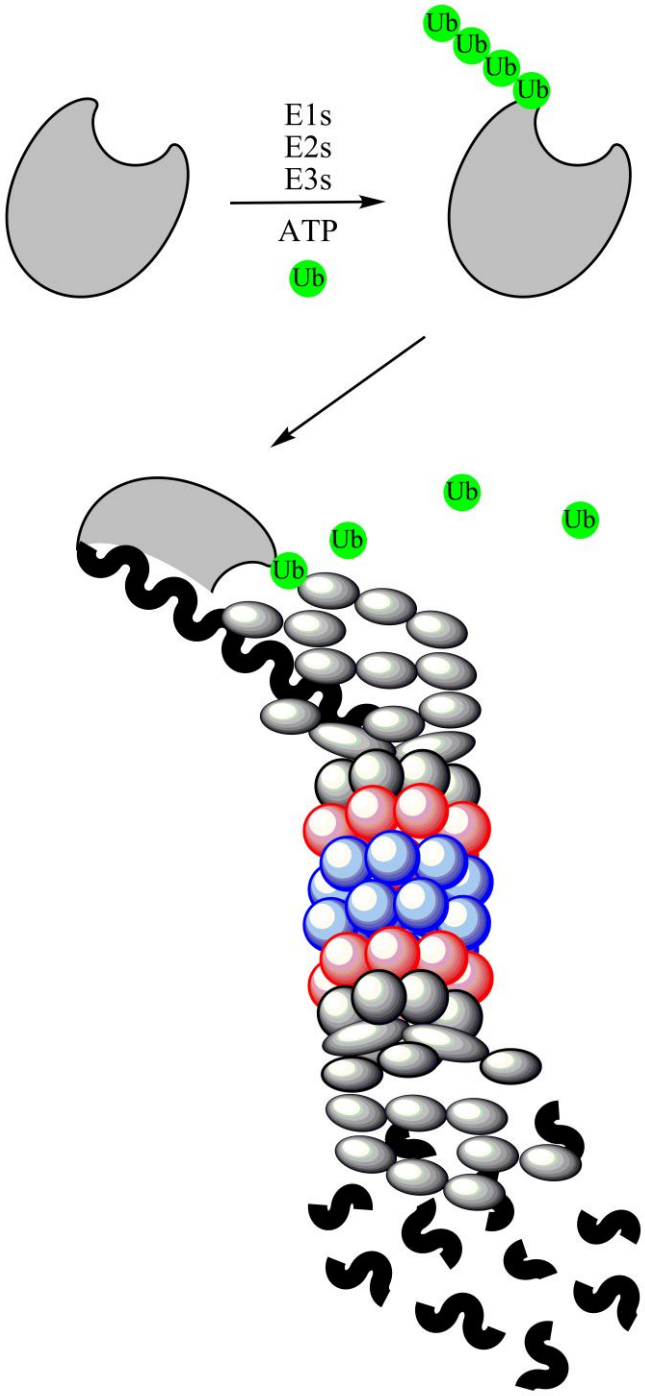


Figure 1.1: The ubiquitin-proteasome pathway. A protein is tagged by multiple ubiquitin molecules (green circles) by a series of enzymes: E1s, E2s, and E3s. The proteasome recognizes these polyubiquitinated substrates, binding, deubiquitinating, unfolding, and degrading the proteins. Short peptide fragments are released by the proteasome to be further degraded by intracellular proteases or used in antigen presentation.

stacked rings. [4] It was proposed to have a variety of protease activities: chymotrypsin-like (CT-L), caspase-like (C-L), trypsin-like (T-L), branched chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAAP). [3, 5, 6]

The importance of the proteasome for normal cell survival was indicated by studies in *Drosophila melanogaster* which identified a mutation in a proteasome subunit as a dominant temperature-sensitive lethal mutation. [7, 8] Later studies showed an additional proteasome subunit mutation with the same dominant temperature-sensitive lethal effect; both mutations function as "poison subunits" which affect the stability and activity of proteasomes at increased temperatures. [8, 9] Additionally, studies in hematopoietic cells from leukemia patients as well as malignant hematopoietic cell lines showed increased expression of proteasomes in a variety of cancers (acute lymphoblastic leukemia [ALL], adult T-cell leukemia [ATL], acute myeloid leukemia [AML], chronic lymphocytic leukemia [CLL], chronic myelogenous leukemia [CML], and Burkitt lymphoma). [10] The authors suggested that, "Thus, abnormally high expression of proteasomes may play an important role in transformation and proliferation of blood cells and in specific functions of hematopoietic tumor cells." [10]

To determine the intrinsic and adaptive function of this complex, probes of its activity would be required. Luckily, biochemists that studied the bovine multicatalytic protease complex had developed substrates for its main proteolytic activities: CT-L, C-L, and T-L. The CT-L activity, cleaving after large, hydrophobic amino acids, is now most commonly examined using N-succinyl-leucine-leucine-valine-tyrosine-7-amino-4-methylcoumarin (Suc-LLVY-AMC), where cleavage after the tyrosine liberates AMC. This cleavage can be observed by excitation at 360nm, allowing for detection of the emission wavelength at 460nM. Likewise, the C-L activity, cleaving after acidic amino acids, is now detected using benzyl-norleucine-proline-norleucine-aspartame-AMC (Z-nLPnLD-AMC) and the T-L activity, cleaving after basic amino acids, is now detected using benzyl-leucine-arginine-arginine-AMC (Z-LRR-AMC). A number of additional substrates have been developed over the years, but these are considered the prototypical substrates for proteasomal hydrolysis.

This method of examining proteasome activity provides a limited amount of useful information. As Peter Kloetzel and colleagues noted in 1995: "We conclude that the hydrolysis of short fluorogenic peptides does not adequately describe the cleavage of peptide bound in larger peptides by the 20S proteasome and that the decision on the cleavage site … is not simply

dependent on the residue in the P1 position." [11] Nevertheless, this technique remains a popular one to describe proteasome activity.

To better understand the role of this protease complex in normal and disease states, inhibitors of this complex which could be utilized to conclusively determine its function would be required. Marian Orlowski and colleagues produced the first peptide aldehydes (Z-LLF-CHO, Ac-LLnL-CHO, and Ac-LLM-CHO) which inhibited the CT-L activity of the bovine multicatalytic protease complex as slow-binding, reversible inhibitors. [12] At higher concentrations, these small molecules were also found to inhibit the T-L and C-L activities of the complex, providing biologists with the final tools necessary to examine the proteasome hypothesis. [12] In 1994, Alfred Goldberg's group published a paper in Cell entitled, "Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules." [13] For the first time, they showed that the majority of cellular proteins were degraded by the proteasome and that this pathway was important for the proper functioning of the immune system, as it created the peptides necessary for major histocompatibility complex (MHC) class I molecules. [13] Thus began a flurry of work to further define and examine the proteasomal structure, assembly, activities, and functions.

B: Proteasomes and their catalytic beta subunits

Mammalian proteasomes are now known to contain catalytic beta subunits which degrade polypeptides. Early studies examined these protein sequences and hypothesized that these subunits may be synthesized as precursors, and later processed to the active form, likely by cleavage at Gly^{-1}/Thr^{1} leaving an N-terminal threonine to serve as a nucleophile. [14] At least two proteolytically active subunits were known at the beginning of the 1990s, which we now call Y/β1 and X/β5. Around this time, a second set of proteins named low molecular mass proteins two and seven (LMP2 and LMP7) were characterized by sequence alignment; they were hypothesized to be proteolytic subunits, also synthesized as precursor proteins but present in processed forms in intact proteasomes. [14-16] These LMP proteins were found as gene pairs, with LMP2 and antigen peptide transporter 1 (TAP1) or LMP7 and TAP2 in close proximity. [17] TAPs are ATP-binding cassette (ABC) transporters which take peptides from the endoplasmic reticulum (ER) to the cell surface for class I antigen presentation. By looking at sequence homology and exon/intron pairing, the authors concluded that LMP7/TAP2 was the original gene pair, duplicated to produce LMP2/TAP1. [17] These genes are well conserved, as the

cluster originated prior to the human/mouse/rat species divergence, with LMP2 sharing 84% DNA sequence and 88% protein sequence homology between humans and mice. [17] Both gene pairs have putative interferon-stimulated response elements in their promoter regions, and their expression is stimulated by interferon experimentally. [11, 16-23]

Further studies of the LMP proteins found they were able to replace the "constitutive" proteasome subunits. LMP2 replaced β1 and LMP7 replaced β5, thus current nomenclature calls them β1i and β5i, respectively. [22] Interestingly, β1 and β1i only share 76% sequence similarity, but their expression levels seemed to be inversely correlated. [22, 24] Experiments were undertaken to determine what changes may occur when one set of subunits was replaced by the other. They found that adding β1 to cells increased the C-L activity but did not affect the T-L or CT-L activity, while adding β5 decreased the CT-L and T-L activity but had no effect on the C-L activity. [24] Conversely, the addition of β5i increased the T-L and CT-L activity, while not affecting the C-L activity; the addition of β1i decreased the C-L activity and increased the T-L activity, but did not affect the CT-L activity. [21, 24, 25] Notably, adding β5 increased expression of β1, and *vice versa*, suggesting these subunits incorporate into proteasomes cooperatively. [24]

The activity change seen above paired with the proximity to the TAP genes, lead many to theorize that these LMP subunits may form a different kind of proteasome, which was dubbed an immunoproteasome, to produce antigens for MHC class I presentation. MHC class I molecules are found on all nucleated cells and function to display small, internally produced peptides to T cells. If the peptides presented on the cell surface contain foreign proteins, known as antigens, the cell will be attacked by the immune system; otherwise, the cell is ignored. It had been shown that the TAP proteins were required for antigen presentation by MHC class I molecules. [26, 27] However, work by a number of researchers quickly showed that these alternative catalytic subunits are not obligatory for antigen presentation by MHC class I molecules. [19, 21, 26-28] Still, some suggested that immunoproteasomes may alter the spectrum of peptides produced or amplify specific proteolytic activities favorable for antigen presentation. [19, 21, 25, 27, 29] Nevertheless, the vast majority of peptides presented by MHC class I molecules are generated by some form of the proteasome. [30]

Further studies of the LMP subunits found that their expression could be induced by treatment with interferon-γ. [22] This work demonstrated that interferon-γ causes the expression of the

"inducible" proteasome subunits (β1i and β5i), thus the increase in the inducible subunits led to a decrease of proteasomes containing the constitutive subunits. [22] However, the amount of β1i present in a cell after treatment with interferon-γ increases to varying degrees in a cell line-dependent manner. [28] Transfection of β1i and β5i likewise mimic the changes in proteolytic activity and products produced by treatment of cells with interferon-γ, strongly suggesting that interferon-γ produces these changes by upregulating these two proteins. [21] Knocking out signal transducer and activator of transcription 1 (Stat1) reduces the constitutive expression of immunoproteasome subunits, suggesting it might play a major role in their normal expression. [31] Thus, it is important to note that interferon-γ is essential for upregulation of immunoproteasome subunits, but not their constitutive expression. [31]

Not long thereafter, the third pair of exchangeable subunits was identified: β2/Z and β2i/MECl-1/LMP10. [32-34] Like the other inducible beta catalytic subunits, β2i can replace β2 in humans and mice, the β2i gene shares 89% identity between the species, and β2i is inducible by treatment with interferon. [32-37] Interestingly, the size of the β2i and β2 genes differ significantly, although both have eight exons and equivalent exon-intron boundries, suggesting they likely arose by duplication from a common ancestor. [36] Later studies which examined β2 found that its expression levels, like those of β1, increased upon β5 overexpression. [38] Additionally, a mutation of β2i which abrogates its catalytic activity causes loss of T-L activity, which is not a required proteasomal activity in rabbit tissues. [39, 40]

As demonstrated above, the beta subunit genes are scattered throughout the genome, suggesting the stabilization of these proteins by incorporation into proteasomes; this would permit the rapid turnover of unincorporated subunits and provide a mechanism by which the subunits could be regulated in a cooperative manner. [41] In addition, studies show that the MHC class I expression deficiencies of a number of tumor cell lines could be overcome by treatment with interferon-γ. [42, 43] This implies that such alterations in the protein expression patterns of malignant cells are due to regulatory or epigenetic changes, rather than direct genetic mutations.

Additionally, the net effect of the incorporation of these inducible subunits in proteasomes may depend on the overall proteasome beta catalytic subunit composition in a cell or tissue. [44] With regard to the these proteins, it has been found that high mRNA levels do not always correlate with high protein levels, and the expression of β1i and β5i at the protein level varies

substantially between cell lines and tissues. [15, 28, 45, 46] In mice, the mRNA levels of β1i and β5i are high in the thymus, spleen, and lung but absent in the brain and muscle, while protein expression is highest in the spleen. [15, 47] Likewise, β1i and β5i are constitutively expressed in all thymic antigen presenting cells in perinatal rats. [48] Moderate mRNA and protein levels of β1i and β5i are seen in mouse liver, kidney, and heart; in general, β1i and β5i are evenly distributed between the cytoplasm and nucleus in mouse liver. [15, 47, 49] Interestingly, treatment with interferon-γ can induce immunoproteasome subunit expression in immunoprivileged organs of mice, such as the lens of the eye and the brain. [46, 50]

In humans, the inducible subunit β1i mRNA levels are undetectable in fibroblasts, as well as smooth muscle, colon epithelial, and brain cells. [16] β1i was not found in fetal livers and β5i was seen in only one-third of the samples examined. [51] However, β1i and β5i were seen in all adult liver samples at varying intensities. [51] Moreover, β1i, β2i, and β5i are expressed in human fetal and adult thymal tissues such as the stroma, epithelium, and macrophages of the cortex and medulla, but not in immature thymocytes. [52] A C-terminal tagged version of β1i (LMP2-GFP) was found to be incorporated efficiently in proteasomes located through the cytoplasm and nucleus of human fibrosarcoma cells. [53]

C. Crystallographic and structural studies of proteasomes

Thermoplasma acidophilum is an archaebacterium which contains a very primitive form of the proteasome. This was ideal for the initial studies of proteasomal crystal structure because of its simplicity, yet this archaebacterial proteasome was similar enough to be used to model the proteasomes of eukaryotes. Its proteasome contains two distinct subunits, called alpha and beta, which are composed of a core of two five-stranded antiparallel beta sheets with three alpha helices above and two below. [54] The alpha and beta subunits have significant sequence homology, suggesting they probably evolved from a common gene. [54] The crystal structure of the *T. acidophilum* proteasome depicts a complex with 72 point symmetry, a molecular weight of 673 kDa, and the barrel shape seen in earlier electron microscopy experiments. [54] There are four rings, two alpha rings on the ends with seven subunits each and two beta rings in the center, also with seven subunits each. [54] The alpha subunits contain two putative nuclear localization sequences which were shown to be accessible to solvent in the crystal structure. [54] Additionally, the tight packing of the barrel structure should prevent access to the catalytic

core from any location other than the central channel, where the disordered N-termini of the alpha subunits could permit the entry of polypeptides. [54]

Interestingly, the first eleven residues of the beta subunits were not present in the crystal structure, but Lys^{33} and Glu^{17} create a salt bridge in close proximity to Thr^1, which may form a catalytic triad. [54] These first eleven amino acids are known as the propeptide of the beta subunit, and their loss is necessary to produce a catalytically active subunit. Later studies indicated that these beta catalytic subunit propeptides are not required for the assembly of the *T. acidophilum* proteasome. [55] Treatment of proteasomes with the aldehyde inhibitor Ac-LLnL-CHO modified all of the beta subunits by formation of a hemiacetal with Thr^1, but did not cause a conformational change in the protein. [54] Mutational studies showed that while $Thr^1 \rightarrow Ala^1$ mutants do not bind the proteasome inhibitor NLVS (4-hydroxyl-5-iodo-3-nitrophenyl acetate-leucyl-leucyl-leucine vinyl sulfone), $Thr^1 \rightarrow Ser^1$ mutants did, suggesting the side chain hydroxyl of the first amino acid is important for the proteolytic function of these proteins. [56]

With this structure in hand, scientists next examined the yeast proteasome crystal structure. They found a similar quaternary structure when compared to the *T. acidophilum* proteasome, with four stacked rings but only two-point symmetry. [57] This reduction in symmetry is due to the existence of seven distinct alpha subunits and seven distinct beta subunits in yeast, which have the same secondary structure as seen in *T. acidophilum,* but form rings containing one copy of each distinct subunit ($\alpha_{1\text{-}7}/\beta_{1\text{-}7}/\beta_{1\text{-}7}/\alpha_{1\text{-}7}$). [57] Structurally, these proteasome subunits resemble N-terminal nucleophile hydrolases (Ntn-hydrolases). [57]

In yeast, only three of the subunits in each beta ring have catalytic activity, as demonstrated by their binding to Ac-LLnL-CHO at the Thr^1 hydroxyl: β1 (Pre3), β2 (Pup1), and β5 (Pre2). [19] The propeptides of these catalytically active subunits are processed between Gly^{-1} and Thr^1, with loss of Thr^1 abrogating the proteolytic activity of the subunit. [57-60] Mutation of the Thr^1 to Ser^1 can maintain proteolytic activity, but, unlike in *T. acidophilum*, this decreases the cellular viability of yeast. [59] While the propeptides of β1 and β2 are not required for proteasome assembly, the propeptide of β5 is necessary for proper incorporation of the subunit, although it can function *in trans* with reduced efficacy to rescue β5 propeptide deletion mutants. [58, 61-63] Interestingly, it appears that the propeptides of β1 and β2 protect the N-terminal catalytic threonine against acetylation by Nat1-Ard1; acetylation blocks the enzymatic activities of these subunits. [62-64] Next, the proteolytic activity of each of these catalytic beta subunits was

examined. Mutational studies indicated that β1 had C-L activity, β2 had T-L activity, and β5 had CT-L (perhaps also some T-L) activity, but only the loss of β5 activity had a significant effect on cell viability and increased the levels of proteasomes present in cells. [57-62] Another group suggested that BrAAP activity could be assigned to both β1 and β2. [60]

Unlike the *T. acidophilum* proteasome, the other beta subunits in the yeast proteasome are catalytically inactive. β3 and β4 are not processed, while processing of β6 and β7 by their catalytically active neighboring subunit leaves an eight to ten amino acid cap at the N-terminus; the processing of β1, β2, and β5 occurs via intrasubunit autolysis and only requires dimerization of the proteasome halves. [57, 61, 64] β7 is important for this dimerization process, as its C-terminal tail interacts with the other half proteasome to bring the two together in the proper alignment. [65] Nevertheless, all subunits are important, and mutations in any can be detrimental, as failure in assembly or processing is often more harmful to cells than proteolytic inhibition. [61] The makeup of the substrate binding pocket is dependent on residue 45, found at the bottom of the binding pocket; however, adjacent subunits of the beta ring also contribute to the final binding pocket characteristics. [57] Thus, mutations which impinge upon subunit-subunit interactions can abolish or decrease proteolytic activity, interfere with autocatalytic processing of the beta subunits, or destabilize the fully assembled proteasome. [57, 58, 61, 66]

The core particle itself is autoinhibited by the alpha subunits, specifically their C-terminal tails, which cap the ends of the barrel and can prevent substrate entry. [67] α3 has an especially important role in this process, as its tail projects across the axis of symmetry, and this inhibition is relieved by the binding of a regulatory particle or treatment with sodium dodecyl sulfate. [67, 68] Additionally, hydrophobic peptides have been suggested to stimulate proteolysis by opening a channel in these alpha ring tails. [69] Alpha rings are formed with the help of dedicated chaperones Poc3/4 (Pba3/4) while proteasomes in general also require the assistance of Poc1/2 and Ump1p for normal cooperative assembly. [65, 70-72]

D. Proteasome structure, assembly, and localization in mammals

To begin to understand the assembly of human proteasomes, researchers looked to mice for detailed mechanistic answers. Since mouse ubiquitin-proteasome pathway proteins generally have high homology with human proteins, and mice express both constitutive and immunoproteasome subunits, they are a better model for the human proteasome assembly

process than yeast. From mouse studies it was determined that proteasome assembly is an irreversible process which requires continuous protein synthesis. [22, 73, 74] The first step in proteasome assembly is the formation of an alpha ring. [74] Mouse proteasome assembly chaperones-3 and -4 (mPAC3/4) are homologous to yeast Poc3/4; they assist in the formation of alpha rings and remain as some of the beta subunits are added. [70] Beta subunits are added directly to the alpha ring as unprocessed propeptides to form precursor complexes (13S-16S). [73, 74] This is the step in which homologous subunit exchange may occur, as inducible proteasome subunits replace constitutive ones; proteassemblin, a homologue of yeast Ump1p, steps in at this point to help chaperone the assembly process. [22, 73, 75]

These 13-16S precursor complexes are lost as 20S proteasomes are formed, a process which occurs in the cytoplasm and may continue with the association of a 19S regulatory cap to form a 26S proteasome. [73, 74] It is important to note that processed catalytic subunits are found only in 20S proteasomes, as subunit processing and complete proteasome assembly are concurrent processes, and it is only after cleavage of the propeptide sequence that beta catalytic subunits are proteolytically active. [22, 73, 74] 11S and 19S caps are ATP-dependent enzymes which facilitate the deubiqutination and entry of substrates into the 20S catalytic core. [73] The amount of these caps is rate limiting, so association with 20S core particles is rapid and leaves many 20S proteasomes without a cap. [73, 76] Fully assembled 26S proteasomes, containing a 20S core and a 19S cap, can thus begin efficiently degrading proteins.

In humans, the process of assembling proteasomes proceeds in a similar manner. Human proteasomes are composed of two symmetrical halves, each holding a single copy of each alpha and beta subunit in their assigned position within their ring. [77] A number of chaperones are involved in this process. First, PAC1/2 form a heterodimer to interact with α5 and α7, assisting in the formation of an alpha ring. [78] The human analogue of yeast Ump1p and mouse proteassemblin (95% identity), POMP (PrOteasome Maturation Protein), interacts with the ER membrane and the alpha ring. [75, 79, 80] The levels of POMP mRNA increase upon treatment with interferon-γ, likely resulting in increased protein levels which assist with more rapid proteasome assembly. [79] POMP and the PAC3/4 dimer (homologues of yeast Poc3/4) then assist in the recruitment of beta subunits onto the alpha ring. [70, 80] Unlike in yeast, human proteasome propeptides are not essential for the correct positioning of subunits within the core particle. [81] However, the β5 propeptide directs proteasome assembly to encourage the

cooperative assembly of constitutive proteasomes and immunoproteasomes, rather than intermediate proteasomes. [79, 82] Additionally, having a propeptide, even one of another subunit, is important for the incorporation of the β5 and β5i subunits, which are the rate limiting step of proteasome assembly. [82]

A knockdown experiment undertaken using siRNA in human embryonic kidney cells suggests that beta subunit assembly begins with β2 binding to an alpha ring associated with POMP, which requires the β2 propeptide and C-terminal tail. [83] This is followed by the addition of β3, which is coupled to the loss of PAC3. [83] The addition of β4 creates the 13S half proteasome, a commonly seen assembly intermediate. [83] β5 is then added and, assuming its propeptide is present, β6 incorporates. [83] Apparently, β1 can incorporate at any time after β2/3, but its incorporation is a prerequisite for the addition of β7. [83] Additionally, there is an interesting correlation between β1i and β2i incorporation, with some studies suggesting β1i enhances β2i incorporation, others suggesting the opposite, and some which postulate that the effect is cell-type dependent. [35, 84, 85] Finally, when β7 is added to the ring, which requires the β7 C-terminal tail, the 16S half proteasome is formed. [83] β1 and β1i lose their propeptides while in 16S complexes; however, subunit processing and proteolytic activation do not coincide, as the processing that occurs in 16S complexes is insufficient to provide proteolytic activity, and may require additional factors. [74, 86]

This propeptide processing is a two-step autocatalytic process, requiring the Gly^{-1}/Thr^{1} consensus motif and Lys^{33}. [81, 86, 87] A key piece of evidence supporting this two-step hypothesis is that the β1i mutant, where Ala^{1} is encoded in place of Thr^{1}, results in an N-terminal extension of nine to ten amino acids resulting from cleavage between Gly^{-11}/Ser^{-10} or Ser^{-10}/Phe^{-9}, but does not affect subunit incorporation or complex assembly. [25, 81] A similar result was seen with the same mutation in β2i. [39]

Finally, the tail of β7, along with POMP, assists in dimerizing the 16S proteasome halves. [80, 83] This process can be disrupted by proteasome inhibitor subunit 31 (PI31), which decreases the ability of 16S immunoproteasome to dimerize, although the mechanism for this inhibition is not yet clear. [88] POMP then serves as one of the first substrates of the new, catalytically active 20S proteasome. [80]

While many of the initial studies of constitutive and inducible subunits suggested cooperative incorporation of all three beta catalytic subunits, other studies contradict these findings. [22, 24, 35, 36, 38, 44] In support of the cooperative hypothesis, a study found that treatment with interferon-γ works quickly to reorganize proteasome types, decreasing the number of 20S cores associated with 19S caps and increasing the number of immunoproteasomes, usually within 24 hours. [89] One of the first studies to contradict this hypothesis and suggest the existence of intermediate proteasomes was a study done in mouse macrophages that found β5i to exist in β1i-containing proteasomes as well as in proteasomes depleted of β1i. [28] Another group later hypothesized the existence of eight possible half (16S) proteasomes if all catalytic beta subunits can incorporate independently, which increases to 36 proteasome types if the composition of the two beta rings are independent. [34] This suggests a high level of variability in proteasomes, especially if each intermediate proteasome has different functions or expression levels. Additionally, an increasing level of complexity is present when considering that the 20S cores may associate with regulatory caps. These 19S and 11S caps assemble to form proteasomes containing one, two, both, or no caps. [90] β1i and β5i are found both with and without caps, although more are seen without. [76]

Studies in rats suggest that at least some of these intermediate proteasome cores exist *in vivo*, as six subtypes of proteasomes were identified from rat muscle. [91] While the most abundant type appeared to be constitutive proteasomes, a significant amount was of the intermediate type (β1, β2, β5i and β1/β1i, β2, β5i), and a few were immunoproteasomes. [91] When the authors examined rat spleen, the immunoproteasome type was most prevalent, with some intermediate proteasomes as well (β1, β2i, β5i and β1/β1i, β2i, β5i). [91] Later work suggested the presence of intermediate proteasomes in multiple myeloma (MM), plasmocytoid lymphoma, T-cell lymphoma, non-Hodgkin's lymphoma (NHL), colon adenocarcinoma, and lung adenocarcinoma based on quantitation from enzyme-linked immunosorbent assays (ELISA). [92] Since then, two major intermediate human proteasome subtypes (β1, β2, β5i and β1i, β2, and β5i) have been identified as responsible for the generation of certain clinically-relevant tumor antigens. [93] These subtypes are one-third to one-half of the proteasomes found in human liver, colon, small intestine, kidney, dendritic cells, and tumor cells. [93]

These studies seem to also support the hypothesis that the composition of the two beta rings which assemble to form 20S proteasomes are independent; both β1 and β1i are found in some

subtypes, which was suggested previously in transfected human cells. [53] Additional work in pancreatic cancer cell lines demonstrated high variability of β1i and β2i expression between cell lines, suggesting the proteasome likely forms distinct subtypes in each cell line. [94] Perhaps most significantly, the activity of each subtype against fluorogenic peptide substrates varied considerably, and thus proteasome characteristics of tissues are an average of the activities of the subtypes found in each tissue, even though each type of proteasome may have unique activities and/or functions *in vivo*. [91]

Conclusive evidence for a different kind of intermediate proteasome came in 2007 with the discovery of the thymoproteasome. A thymoproteasome contains β1i, β2i, and β5t, a novel subunit located adjacent to the β5 gene and found exclusively in cortical thymic epithelial cells. [95, 96] β5t has greatly reduced CT-L activity, likely due to its hydrophilic S1 pocket, allowing it to produce the peptides necessary for the positive selection of major and diverse MHC class I-restricted CD8+ T cells. [95, 96] Studies in knockout mice demonstrated the non-lethality of β5t knockouts, but also showed a severely defective response to allogeneic and viral antigens, resulting in lethal influenza infection. [96]

When later studies confirmed the existence of proteasomes containing both constitutive and immunoproteasome catalytic beta subunits, they were found in the cytoplasm, nucleoplasm, and microsomes of HeLa cells. [97, 98] As Marian Orlowski and colleagues noted in 1999, "There is a tendency to assume that substrate binding properties, and therefore specificity of a pair of identical catalytically active subunits, are the same." [99] As the active sites of the proteasome are actually formed by pairs of neighboring beta subunits, one can postulate that intermediate proteasomes may have some effect on the function of identical beta catalytic subunits. [77] One could also postulate that proteasomal cellular localization may be unique between types or have functional consequences.

Proteasomes are generally found throughout mammalian cells, diffusing rapidly throughout the cytoplasm and nucleus, but are absent from the perinuclear region (i.e.: nuclear envelope) and nucleoli. [53, 100] Studies in rodents showed that proteasomes are found in nuclear, cytosolic, and microsomal fractions but not always at the same levels. [73, 101] For example, proteasomes levels in rat liver are ten-fold higher in the cytosol than nuclei and six- to seven-fold higher in the cytosol than the microsomes. [101] Additionally, membrane-bound

proteasome have been localized to the exterior membranes of the smooth endoplasmic reticulum, cis-Golgi, and mitochondria. [101, 102]

Interestingly, proteasomes are only able to diffuse slowly and unidirectionally from the cytoplasm to the nucleus of interphase cells, although they can move about freely during mitosis until the nuclear envelope is restored. [53] While the nuclear distribution of proteasomes is species-specific and varies between higher and lower eukaryotes, human proteasomes are found in speckles, nuclear bodies, nucleoplasmic foci, and diffusely throughout the nucleoplasm in interphase human epithelial cells. [103] These nuclear proteasomes have significant proteolytic activity as they seem to co-localize with and degrade substrate proteins in specific subnuclear foci, such as splicing speckles and PML bodies. [100, 103, 104]

E. Constitutive and immunoproteasome functions

The proteasome is a proteolytic enzyme complex consisting of Ntn-hydrolases and noncatalytic subunits, yet the question of function remains partially unanswered. Generally speaking, the main function of the proteasome is the degradation of proteins. Its function is both this simple and much more complex. Protein degradation is a method by which cells regulate protein function, perhaps best demonstrated by the proteasome-dependency of cell cycle progression. Cellular responses to oxidative and other kinds of stress conditions rely on proteasomes, such that tissues with higher proteasome activity (such as the liver) cope better with stress than those with lower proteasome activity (such as the brain). [105] Oxidized proteins can be degraded without ubiquitin conjugation by any fully assembled form of the proteasome. [106] Likewise, in cells with compromised ubiquitin-conjugating activity, proteasomes preferentially degrade oxidized proteins at nearly normal rates. [106] It is important to note that proteasome complexes are stable during the degradative process. [107]

The overall composition of the proteasome complex affects the activity of the proteasome, as do levels of detergents, ATP, and cytokines, as well as the substrate being degraded. [30, 90, 99, 108, 109] Generally, 26S proteasomes degrade proteins into peptide fragments containing 3-22 amino acids (mean=8), fitting a log normal distribution; the size of these peptides is similar between 26S and 20S rabbit muscle proteasomes as well as *T. acidophilum* proteasomes. [40, 110] However, the 26S and 20S proteasomes from rabbit muscle cleave the same peptides

differently, and inhibition of multiple catalytic beta subunits is necessary to substantially decrease protein proteolysis. [40, 111]

Proteasomes are likewise a major player in the production of peptides for antigen presentation. While the proteasome determines both the N- and C-termini of peptides during cleavage, it is the C-terminus which is more important for antigen presentation. [29, 112] Prior to presentation, however, these peptides may undergo additional processing to produce an antigen of the correct size and sequence. The proteasome is sufficient to generate the correct C-termini of epitopes, but another protease is responsible for generating the correct N-terminus. [29, 112] As one might expect, proteasome inhibition can modulate antigen presentation when the inhibition is partial and selective, or block it completely at high levels. [13, 113]

Additional unorthodox roles for the proteasome have been elucidated in recent years. Proteasomes co-precipitate with eukaryotic initiation factor 3 subunits, ribosomal proteins, and glutamyl-tRNA synthetase in a supercomplex known as the translasome. [114] Proteasomes also influence the transcriptional machinery as well as histone methyltransferases. [115] More interestingly, unequal partitioning of proteasomes between daughter cells during metaphase in T lymphocytes is responsible for the unequal distribution of proteins into daughter cells, promoting unique differentiation events in each. [116] Increased expression of the proteasome subunit β5 has been shown to enhance resistance to oxidative stress and increase the number of doublings prior to senescence. [38] Perhaps it is not surprising that increased expression of 26S proteasome genes is correlated with tumor progression and metastasis, accurately predicting clinical outcomes of breast cancer patients. [117]

The functions of immunoproteasomes, on the other hand, are less well defined. Immunoproteasomes are also protective against oxidative stress and degrade oxidized proteins efficiently. [118] In a human lymphoblastoid cell line, mutating β5i, and thereby inhibiting its CT-L activity, has a significant effect on the growth rate of cells, which suggests this activity is required for survival. [79] In general, immunoproteasomes seem to degrade substrates more rapidly than constitutive proteasomes. [89]

26S immunoproteasomes produce peptides of 3-22 amino acids (mean=7-8) fitting a log normal distribution. [110] Compared to constitutive proteasomes (from rabbit muscle),

immunoproteasomes (from rabbit spleen) cleave a hydrophobic substrate [Z-GGL-AMC] 50% faster, a basic substrate [Boc-LRR-AMC] 100% faster, and an acidic substrate [AcYVAD-AMC] at 20% of the rate of proteasomes. [110] This loss of acidic proteasome cleavage can be most directly linked to the replacement of β1 by β1i. [21, 24, 25] Homology studies suggest that the altered amino acid composition of the S1 pocket of β1i makes it more apolar and constricted which should reduce the C-L activity and promote the CT-L activity of this subunit. [57]

Some more exotic roles for immunoproteasomes have been discovered as well. They have a role in proper retinal function in mice, as retinal function is disturbed in β2i/β5i knockout mice. [119] β1i is involved in human trophoblast invasion, the process by which a human embryo implants and interacts with the maternal uterus. [120] Proteasomes containing β1i are also recruited by steroid receptor coactivators and enhance estrogen receptor-mediated transcription, both at the initiation and elongation steps. [121]

When it comes to antigen presentation, immunoproteasomes have varying levels of efficacy in producing the correct antigenic peptide. It is well understood that the importance of any particular catalytic subunit for antigen presentation is epitope dependent. [25, 85, 109-111, 122, 123] Dendritic cells, a set of antigen presenting cells which constitutively express the immunoproteasome, are incapable of producing antigenic peptides generated by the constitutive proteasome, a finding that has implications for vaccine development. [124] The products of insulin-like growth factor 1 cleavage vary significantly when comparing proteasomes to immunoproteasomes of rabbits. [110] An analysis of ten antigens from melanoma shows that four were presented more efficiently after immunoproteasome induction while six were presented less efficiently. [124] For example, the immunodominant epitope of the Ova protein is produced by immunoproteasome cleavage of Ova 11% of the time, but it is only produced 6% of the time when Ova is cleaved by the constitutive proteasomes. [110] Likewise, overexpression of β1i, β2i, and β5i greatly improved the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope, although neither β1i or β5i was required for clearance of this viral infection in mice. [122, 123] β5i, however, is essential for the survival of mice after infection with *Toxoplasma gondii*. [125] Addition of catalytically inactive β1i (T21A) reduces the processing and production of some antigenic peptides, but may enhance that of others. [25] Similarly, the production of these antigenic peptides, but not other parts of the peptide presentation process, can be blocked by inhibiting the immuno/proteasome. [30]

Bioinformatics analysis shows that immunoproteasomes cleavage is more amino acid specific than constitutive proteasome cleavage, as immunoproteasomes cleave after fewer amino acids residues. [126] Interestingly, the use of the highly abundant amino acid leucine as a degradation signal allows the immunoproteasome to degrade proteins with a similar efficiency when compared to constitutive proteasomes. [126] The increase in CT-L activity found in immunoproteasomes allows for the generation of significantly more potential MHC class I ligands than constitutive proteasomes; however, these changes in proteasome activities do destroy some antigenic peptides. [109, 124, 126]

Interferon-γ also plays a major role in the efficient production of antigens. It stimulates the production of leucine aminopeptidase (LAP) and endoplasmic reticulum associated protein 1 (ERAP1), proteins which trim the N-termini of peptides. [29, 127] ERAP1 trims these N-termini sequentially, but not processively, and cannot trim peptides shorter than 8 amino acids. [127, 128] Additionally, interferon-γ induces the expression of the subunits of the 11S regulatory particle, which enhance antigen presentation independently of any changes to the beta catalytic subunits. [129]

A way to begin to examine the possible function of immunoproteasomes and their beta catalytic subunits is to knock out these subunits in mice and explore the phenotypic results. β1i, β2i, and β5i knockout mice have been generated and found to be without gross physical abnormalities. [44, 47, 130] β5i knockout mice were the first to be reported; they show reduced MHC class I expression on cell surfaces, present some peptides less efficiently, but have normal numbers of T and B cells. [44, 131] The effect of β5i on MHC class I expression is dominant, as seen in the case where β5i knockout mice die from *T. gondii* infections which are not lethal to wild type mice. [125, 131] While loss of β5i is deleterious to the infectious response, it seems to be beneficial in a mouse model of colitis, a disease characterized by intestinal inflammation. β5i knockout mice recover more quickly from dextran sulphate sodium-induced colitis and have less severe disease, likely due a reduction in the proinflammatory T cell and mucosal immune responses in the colon of β5i knockout mice. [132]

On the other hand, β1i knockout mice were reported to have reduced levels, but not altered ratios, of CD8+ T lymphocytes in the blood, spleen, and thymus. [47, 122, 131] Similarly, their ability to present antigens and activate T cells in response to infection was lessened. [47] This may be due to the fact that loss of β1i has been shown to alter the overall composition of the

proteasome, although β5i incorporation is not significantly affected. [47] These mice are more susceptible to *T. gondii* infection than wild type mice, but less so than β5i knockout mice. [125] β1i knockout mice also have smaller hearts and lower heart rates than wild type mice. [49, 133] Further studies of the β1i knockout mice showed that their ambulation and cognitive function were the same as control mice of the same background, but they had a higher body weight and degree of motor function. [49, 133, 134] One study suggests that the increased motor function observed in patients with Parkinson's and Huntington's disease may relate to changes in proteasome subunit expression in the brains of these patients. [134]

Proteasome activity in the spleen and liver, but not the muscle and brain, was altered in β1i knockout mice. [47] They thus assemble mixed proteasomes in a tissue specific manner, which likely compromises antiviral antibody responses. [135] Moreover, levels of β5i and β2i were significantly reduced in β1i knockout hearts, resulting in lower proteasome activities worsened by ischemic preconditioning. [133] This lack of cardioprotection in knockout animals can be traced to their inability to inactivate phosphatase and tensin homolog (PTEN) by degradation in the heart. [49, 133] The cardiomyopathy seen in the knockout mice is similar to that observed in type 2 diabetic hearts, suggesting immunoproteasome subunit expression may be responsive to blood insulin levels. [49] Interestingly, female β1i knockout mice develop spontaneous uterine neoplasms as they age, while β1i/p53 double knockout mice have a small but significant reduction in survival. [136, 137]

β2i knockout mice have fewer CD8+ splenocytes than wild type mice and show a reduction of cytotoxic T lymphocyte response to certain epitopes in response to infection with lymphocytic choriomeningitis virus. [130] While β2i knockout mice incorporate β5i efficiently, β1i incorporation is reduced, suggesting cooperative assembly of these two subunits. [130] Double knockout mice of β2i and β5i were also created and found to possess fewer CD8+ T cells in the spleen. [131] After subjecting these mice to whole body irradiation and bone marrow transplantation, multiorgan autoimmunity develops with the lack of immunoproteasome subunits in the target tissues in a causative role. [138]

F. The ubiquitin-proteasome pathway and immunoproteasomes in disease

As one might imagine from their important role in normal cellular functions, the ubiquitin-proteasome pathway and its constituents play distinct and important roles in a variety of

disease states. Pure numbers would suggest these enzymes are important, even excluding their function as regulators of cellular proteins. Current estimates show eight E1 enzymes, approximately 40 E2s, and almost 100 DUBs in five families. [139] A single subtype of E3 ligases alone exceeds the total number of kinases in cells. [139] As the majority of cellular proteins proceed through this pathway prior to degradation, the proteasomes control a number of important proteins implicated in disease. Immunoproteasomes specifically have been implicated in cancers as well as autoimmune, inflammatory, neurological, and infectious diseases. Unfortunately, some of these studies rely on mRNA upregulation to suggest the protein is important to the disease process, though studies have clearly shown that upregulation of proteasome subunit mRNA does not always translate to increased protein expression. [45, 46]

Perhaps the most controversial link between immunoproteasomes and autoimmune disease came from Faustman's group, who suggested that β1i plays a role in the reduction of NF-κB activation in a mouse model of diabetes (NOD), a link strongly disputed by the groups of Ploegh and Monoco. [140-143] Resolution of this issue awaits a truly selective β1i inhibitor and a group interested in wading into such rough waters. Other, better accepted links between autoimmune diseases and immunoproteasomes are found in the more recent literature. Circulating proteasomes are detected at significantly higher levels in patients with active systemic autoimmune diseases (autoimmune myositis, systemic lupus erythematosus (SLE), primary Sjögren's syndrome, rheumatoid arthritis (RA), and autoimmune hepatitis) compared to healthy controls and are thought to correlate with other markers of disease severity/activity, possibly representing the extent of systemic cellular damage. [144] Two patients were shown to have β5i present in their circulating proteasome samples. [144] Another study examining primary Sjögren's syndrome found upregulation of β1i, β2i, and an 11S subunit (PA28α) mRNA levels, but downregulation of the β1i protein when compared to normal controls. [45] Interestingly, the other autoimmune patient tissues examined in this study had increased β1i subunit expression compared to normal controls. [45] Expression of β1i has also been correlated with Hashimoto thyroiditis and psoriasis in patients. [145, 146] β5i expression is correlated with Hashimoto thyroiditis and type I diabetes. [49, 138, 145]

Due to the role of immunoproteasomes in antigen presentation, research into the effects of the immune system and infection on proteasome composition is expanding. In the area of vaccine

development, researchers are finding that in those proteins for which the immunodominant antigen(s) are not produced by the immunoproteasome (the major proteasome subtype in antigen presenting cells), vaccines should contain the immunodominant peptide rather than the whole protein. [124] A number of viral infections utilize this weakness of the immune system and hijack the cellular processes by which immunoproteasomes are produced to avoid detection by the immune system. [147] Specifically, while infection of mice with the lymphocytic choriomeningitis virus in the brain leads to upregulation of immunoproteasome catalytic subunit mRNA, few active and assembled proteasomes are found there, likely due to post-translational regulatory mechanisms. [46] Mouse cytomegalovirus actually makes a protein (M27) which inhibits Stat2 and disrupts interferon-γ signaling, leading to the blockage of immunoproteasome subunit transcription. [147] Human cytomegalovirus can also block the production of immunoproteasome subunits. [147] Interestingly, one study suggests that type I interferons (alpha and beta, predominately) may actually be more important for the upregulation of immunoproteasome subunits in infections than the classically important interferon-γ. [148] Much still remains to be understood about the role of immunoproteasomes, constitutive proteasomes, and intermediate proteasomes in the immune response to infection.

Neurological disorders are perhaps the most surprising pathology for which immunoproteasome expression has been indicated, considering the immunoprivileged status of the brain. The link between the brain and immunoproteasome subunit expression was first suggested in studies with knockout mice, and later examined in other rodents. [134] In a conditional mouse model of Huntington's disease, β1i and β5i but not β2i were upregulated in the cortex and striatum after significant neuropathy had occurred. [149] Further examination found β1i upregulation in all cortical neurons displaying the ultrastructural features of degeneration. [149] A more mechanistic study in a rat model of amyotrophic lateral sclerosis (ALS) showed that immunoproteasome upregulation was a compensatory response to the disease, such that blocking immunoproteasome induction is quite deleterious in this model. [150] In animal models of experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS), upregulation of β1i, β2i, β2, and β5 was seen. [151] Unlike the ALS model, however, treatment with a proteasome inhibitor alone, or in combination with lysosomal inhibition, seems to be a potential therapeutic strategy. [151]

When examining patients with neurodegenerative conditions, links have been found between immunoproteasome subunit expression and disease pathology. For instance, in grade four Huntington's disease patients, β1i and β5i expression increased in the cortex and striatum when compared to age-matched controls. [149] Additionally, β1i expression in the hippocampus and cerebellum, specifically in the neurons and astrocytes, was higher in patients with Alzheimer's disease compared to non-demented elderly. [152] The young show no such expression of β1i in these parts of the brain. [152] Another study looking at risk factors for MS in Italians found females with a certain allele combined with a β1i polymorphism have a lower risk of developing MS, possibly due to fewer autoreactive T cells. [153] This lends support to further investigations of the role of immunoproteasomes in neurological disorders.

Another group of pathologies which upregulate immunoproteasomes are inflammatory diseases. In general, immunoproteasomes play a role in the cellular damage response to stresses such as interferons, lipopolysaccharide, arsenic trioxide, nitric oxide, and heat by clearing the cells of damaged proteins. [89] Immunoproteasomes actually clear oxidatively and otherwise damaged proteins more quickly than constitutive proteasomes. [89] Thus, an expected effect of diseases associated with inflammatory cytokines is immunoproteasome upregulation, although this may not always be beneficial. In inflammatory bowel disease (IBD), immunohistochemistry detected increased β1i expression in diseased and histologically normal tissues from IBD patients compared to healthy control patients. [154] The correlation between β1i expression and histological grade and/or intestinal pathology of these patients was highly significant. [154] Similar results were found in the livers of patients with chronic alcoholic hepatitis and hepatic cirrhosis. In these patients, β1i and β5i immunohistochemical staining was highly significantly increased overall, as well as in the nucleus, when compared to normal tissues. [51] In an inducible model of ulcerative colitis, a type of IBD, β5i knockout mice recover more quickly and have less severe disease than control mice, possibly due to a reduction in the proinflammatory response of the mucosal immune cells. [132] Inhibition of the proteasome using small molecule drugs gave a similar or better effect than β5i knockout, although high doses of proteasome inhibitors produced severe, drug-mediated side effects. [132]

Some diseases are associated with changes in the amino acid sequence of proteasome catalytic beta subunits rather than their expression levels. For instance, hypersensitivity pneumonitis, an inflammation of the lung due to the inhalation of organic particles by a susceptible patient, is

very significantly associated with β5i and β1i polymorphisms. [155] More recently, single polymorphisms in β5i were found in two different inflammatory diseases: JMP, an autosomal-recessive syndrome presenting with joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced childhood-onset lipodystrophy and Nakajo-Nishimura/JASL, characterized by joint contractures, muscle atrophy, fever, rash, and loss of adipose tissues. [156-158] These mutations have been shown to disrupt the tertiary structure of the β5i protein, reducing both its incorporation into proteasomes and its catalytic activity. [156-158] As more groups begin to explore this portion of the proteome, additional correlations will likely be discovered.

Ubiquitin-proteasome pathway dysfunction is observed in most, if not all, cancers. [159] As the components of this pathway are so numerous, it is unlikely that there will be any one target which will be generally therapeutic. However, proteasomes may serve as an indicator of the severity or activity of diseases, such as autoimmune myositis, SLE, primary Sjögren's syndrome, RA, autoimmune hepatitis, and cancers. [144, 160] In case studies, researchers were able to correlate the disease process with the levels of circulating proteasomes, which may represent the extent of systemic cellular damage. [144, 160]

Circulating proteasome levels are also important in the case of monoclonal gammopathy of undetermined significance (MGUS), a disease that is best described as a milder version of MM. Specifically, the levels of circulating proteasomes are higher in MGUS patients compared to healthy controls, and are higher in MM patients than MGUS patients. [160] Additionally, increased concentration of circulating proteasomes correlates with advanced MM and is an independent prognostic factor for MM. [160] As proteasome inhibitors are currently indicated primarily for MM, this finding represents an interesting and novel way to track progression of the disease and also suggests the importance of the proteasome in the disease process.

As Hanahan and Weinberg note in, "Hallmarks of cancer: the next generation" an update of their seminal review article from 2000, two of the emerging hallmarks of cancer are tumor-promoting inflammation and evading immune destruction. [161, 162] The regulation of the immunoproteasome can be linked to both of these hallmarks. Immunoproteasome catalytic subunit expression is often important for cellular responses to inflammation, due to molecules such as interferons, tumor necrosis factor-α, nitric oxide, and lipopolysaccharide promoting immunoproteasome formation in cells. [25, 85, 89, 109-111, 122, 123, 148, 163-165] Quite a few studies have examined the role of immunoproteasomes in the antigen generation process of

cancers. The vast majority of studies thus far indicate a correlation between downregulation of the components of the antigen processing pathway and disease progression. It is postulated that loss of these proteins may assist malignant cells in avoiding immunosurveillance. [166]

Dendritic cells are antigen presenting cells that play a major role in immunosurveillance by activating T cells using antigens recovered from tumor cells. These activated T cells can then destroy any cancerous cells which express this antigen. A recent study suggests that reducing the immunoproteasome levels in dendritic cells may induce an antitumor response to cancers containing mainly constitutive proteasomes. [167] Additionally, the expression of β1i and β5i is correlated with the presence of tumor-infiltrating lymphocytes and spontaneous regression in melanomas. [168]

The next pressing question is how cancer cells may be able to prevent the expression of immunoproteasome beta catalytic subunits. A study in melanoma cells utilized histone deacetylase (HDAC) inhibitors to demonstrate that downregulation of these proteins (including β1i and β5i) may occur epigenetically, although changes in mRNA but not protein levels were examined. [169] Additional support for this epigenetic downregulation hypothesis comes from another study showing interferon-γ is capable of inducing expression of β1i, β5i, TAP1, and TAP2 in small cell lung cancer, Whilm's tumors, prostate cancer cell lines, and a neuroblastoma which normally show low to no expression of these proteins. [43] The downregulation of these same four proteins is also seen in the progression of low grade to high grade melanoma as well as in two acute myeloid leukemia patients followed from diagnosis to relapse. [166, 170] Coordinated downregulation of at least seven of these antigen processing machinery components was found in 41% of prostate cancer patients with early recurrence, but only 24% of patients without recurrence. [171]

More specifically, immunoproteasome catalytic subunit expression levels vary in a number of malignancies. β1i, β2i, and β5i expression was significantly lower in high-stage urothelial carcinomas than low-stage ones. [172] In the progression of MGUS to MM, β1i, β2i, and β1 levels decrease, suggesting changes in expression may be predictive of progression from MGUS to MM. [173] The expression of β1i and β5i was reduced or lost in 45% and 48% of esophageal squamous cell carcinomas, respectively, and it was strongly correlated with tumor grade and lymph node status. [174] In prostate cancer, β1i and β5i expression are lower, as detected by immunohistochemistry, compared to normal prostate tissue. [171] β1i and β5i are also

expressed in Hürthle cell adenomas and carcinomas, but with great interpatient and intrapatient variability. [145] Breast cancer patients with high expression of β5i as detected by microarray have significantly shorter survival time. [175] Additionally, knockdown of β5i expression in a resistant breast cancer cell line resensitized the cells to doxorubicin. [175] In the brain, astrocytomas express less β1i than astrocytes, and this downregulation was correlated with grade of the malignancy. [176]

Interestingly, there are a few reports which suggest β1i expression is important for cancer growth and metastasis. In the case of hydatidiform moles, a nonviable but fertilized egg implanted into the uterus, β1i overexpression may play a role in the highly metastatic phenotype sometimes seen in these abnormal growths, which can develop into a carcinoma. [120] Additionally, β1i has been shown to be important for the formation of oncocytes, hypothyroidism, and interferon-γ-induced thyroid growth defects. [145] While these studies seem to complicate the relationship between immunoproteasomes and cancer cell growth, interferon-γ pretreatment was shown to enhance the sensitivity of half of the solid tumor cell lines tested to a proteasome inhibitor drug. [177] This strongly suggests that cells which express immunoproteasomes are sensitive to their inhibition, and thus, proteasome inhibitors may be a viable therapeutic option for certain solid malignancies.

G. The development of proteasome inhibitors

As mentioned above, the first proteasome inhibitors were the peptide aldehydes Z-LLF-CHO, Ac-LLnL-CHO, and Ac-LLM-CHO. [12] Through their library-based approach, M. Orlowski and colleagues found that aromatic residues in the P1 position increase the affinity of the inhibitor for the proteasome's CT-L activity. [12] Later studies showed that peptide aldehydes with leucine as well as phenylalanine in the P1 position were effective inhibitors of bovine pituitary proteasome's CT-L activity. [99] Additionally, the P2 position is often found to be extremely accommodating, as it faces away from the catalytic subunit in yeast proteasomes. [57] Ac-LLnL-CHO was used in crystal studies of the yeast proteasome and found to bind to β1, β2, and β5 at the Thr^1 side chain hydroxyl, likely as a hemiacetal. [57] Further medicinal chemistry efforts yielded Carbobenzoxyl-LLL (MG132) and carbobenzoxyl-LLnV (MG115), which were found to block the degradation of short-lived proteins in yeast (Figure 1.2.A). [178] Later studies with aldehyde inhibitors of specific activities in proteasomes purified from rabbit muscle showed that the CT-L activity is responsible for 11-50%, the C-L activity is responsible for 12-22%, and the T-L

activity is responsible for 3-35% of the degradation of model proteins. [111] Thus, to block more than 50% of the proteasome's activity requires the inhibition of multiple types of catalytic sites. [111] Perhaps most notably, Goldberg and colleagues remark that, "…the rates of proteolysis measured upon inactivation of one site do not simply reflect the contribution of that site but also the capacity of the residual sites to catalyze the degradation of the specific substrate." [111] While the further development of the aldehyde class of proteasome inhibitors has been limited by concerns regarding cross reactivity with cysteine and serine proteases, they have been utilized in a number of the important studies of the proteasome and assisted in the development of selective proteasome inhibitors.

A natural product, lactacystin, was discovered in 1991 to induce the differentiation of neuroblastoma cells (Figure 1.2.B). [179, 180] Later work by the Stuart Schreiber and EJ Cory labs found that lactacystin bound to the catalytic threonine of proteasome catalytic subunits, irreversibly inhibiting its T-L and CT-L activities, and its effect on neurite outgrowth was directly correlated to the degree of proteasome inhibition. [181] While lactacystin prefers the CT-L activity, it can inhibit all three main proteasome activities and modify all six beta catalytic subunits of human proteasomes while not binding reproducibly to other proteins in lymphoblastoid cell lines. [30] Later work showed that the β-lactone of lactacystin was capable of blocking the degradation of short lived proteins in yeast, similar to the effects of MG132 and MG115. [178] Groll and colleagues' yeast crystal structure demonstrated that lactacystin bound to the β5 subunit, where it acetylates the side chain hydroxyl of Thr^1 as a result of the β-lactone ring opening. [57] Additional work that same year showed that lactacystin is a more specific inhibitor of the proteasome than the peptide aldehydes and that it also inhibits interferon-γ-inducible proteasomes. [30] Interestingly, the localization of proteasomes is not affected by proteasome inhibitors, but after 24 hours of treatment, proteasome inhibitors induce a significant increase in proteasomal staining intensity. [100, 103] Later work suggested that the β-lactone pharmacophore also binds to other proteases. [182-184] This, combined with the highly complex synthesis of β-lactones, discouraged further medicinal chemistry efforts. Still lactacystin remains a popular tool for cell and molecular biologists even today.

Another early class of proteasome inhibitors was the vinyl sulfones. The inhibitor N-carboxybenzyl-leucil-leucil-leucil-vinyl-sulfone (Z-LLL-VS) covalently inhibits all three main proteasome activities in purified proteasome as well as in cell lines, although it binds to other

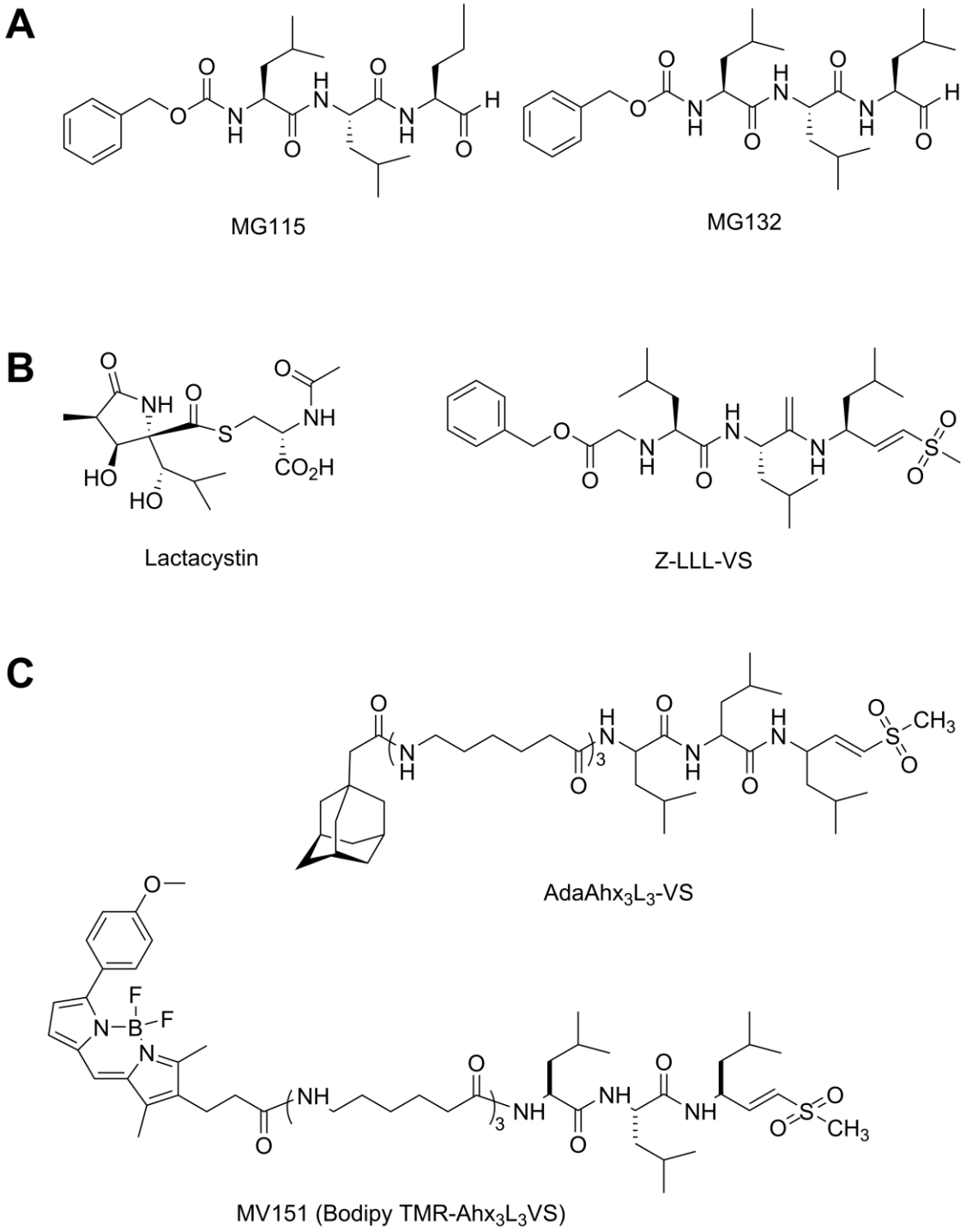


Figure 1.2: First generation proteasome inhibitors. The structures of some early proteasome inhibitors are depicted. **A.** The peptide aldehydes MG115 and MG132. MG132 is still used today as a general proteasome inhibitor. **B.** The natural product proteasome inhibitor lactacystin and synthetic inhibitor Z-LLL-VS. **C.** Molecular probes of proteasome function.

proteases as well (Figure 1.2.B). [56] This class of molecules has been important in studies to understand the binding characteristics of substrates and inhibitors. For example, vinyl sulfone tetrapeptides were used to elucidate the importance of the P4 position for directing binding specificity to active sites, providing more subunit selective inhibitors. [185] In inhibitors with tetrapeptide length or longer, there appears to be less sequence specificity for the catalytic activities than is typically assumed based on the results from small peptide-based substrates and inhibitors. [186] Vinyl sulfones also provided more general proteasome inhibitors, such as adamantaneacetyl-(6-aminohexanoyl)$_3$-(leucinyl)$_3$-vinyl-(methyl)-sulfone (Ahx$_3$L$_3$VS), which binds all six active sites and can be labeled with radioisotopes, biotin, or fluorescent groups (such as MV-151) (Figure 1.2.C). [186] The popularity of vinyl sulfones is limited by their affinity for cysteine proteases, against which they were originally developed, but vinyl sulfones are still used today as molecular probes of proteasomes.

In 1998, dipeptidyl boronic acids were reported to be potent and selective proteasome inhibitors. [187] Boronic ester inhibitors were initially developed to target serine proteases, but boronic acids were shown to be highly selective for the proteasomes over other proteases. [187] Interestingly, boronic acid proteasome inhibitors interact with the proteasome in a competitive but slowly reversible manner. [188] These inhibitors were initially developed in hopes of treating muscle-wasting diseases, but many doctors and biologists were fearful that inhibiting the proteasome clinically would be extremely toxic. [189] The researchers at ProScript quickly decided using these boronic acid proteasome inhibitors as chemotherapeutic agents would be the best way to develop this drug class, as oncology doctors and patients are much more willing to tolerate significant side effects if the drug is effective. [189] Millennium Pharmaceuticals acquired ProScript in 1999 and brought the lead boronic acid proteasome inhibitor into clinical trials in 2000.

In the meantime, a final class of natural product proteasome inhibitors, the epoxyketones, was described in 1999. [190] Eponemycin was shown to bind β1i, β5i, and β5 as well as inhibiting cancer cell growth by causing apoptosis (Figure 1.3.A). [190, 191] Epoxomicin, on the other hand, was shown to bind β2, β2i, β5, and β5i (Figure 1.3.A). [190, 192] It inhibits primarily the CT-L activity of bovine proteasomes at low nanomolar doses, but does not inhibit other proteases such as trypsin, chymotrypsin, papain, calpain, and cathepsin B at concentrations up to 50μM. [192] Crystal structure work was able to show that epoxyketones, epoxomicin

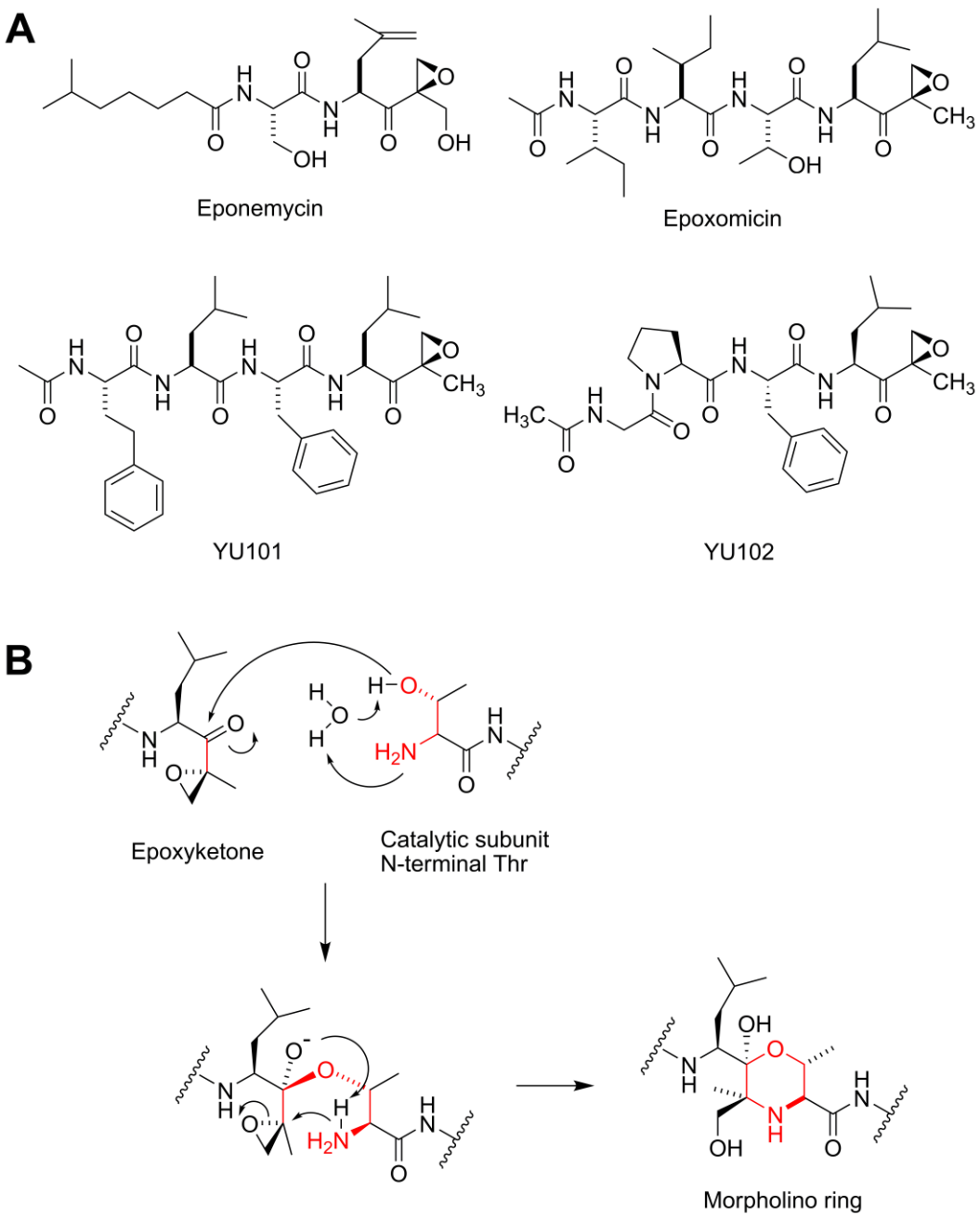


Figure 1.3: Epoxyketone proteasome inhibitors and their mechanism of action. **A.** The structure of the natural product proteasome inhibitors eponemycin and epoxomicin, as well as the synthetic analogues YU101 and YU102. **B.** At top, the pertinent portions of the epoxyketone proteasome inhibitor and catalytic beta subunit are shown. The atoms in red will be part of the morpholino ring. Activation of the threonine by a nucleophile (such as water) allows the threonine to attack the ketone, opening the epoxide ring with simultaneous closure of the morpholino ring.

specifically, form a morpholino ring with Ntn-hydrolases, covalently inhibiting their catalytic activity (Figure 1.3.B) [193]. Epoxomicin helped demonstrate the validity of partial and selective *in vivo* proteasome inhibition as an approach to alter antigen presentation, although the effects were epitope-dependent. [113] Medicinal chemistry efforts with epoxyketones led to the development of YU101, a very potent and selective CT-L activity inhibitor with good anti-inflammatory activity; further efforts provided YU102, a C-L selective inhibitor (at least 50-fold more potent towards the C-L activity than CT-L activity) which did not inhibit protein degradation as a single agent in bovine aortic endothelial cells (Figure 1.3.A). [194, 195] Later medicinal chemistry efforts would be focused on increasing the specificity and potency of these diverse classes of proteasome inhibitors.

H. Proteasome inhibitors as therapeutic agents

Proteasome inhibitors are now changing people's lives in the oncology clinic. In 2000, the first MM patient who received a proteasome inhibitor in a Phase I study, designed primarily to evaluate safety, had a complete remission. [189] Due to such spectacular results as well as close collaboration with the Food and Drug Administration (FDA) and patient groups, bortezomib (MG341/PS-341/Velcade™) was approved in 2003, before the final Phase III trial was complete. Bortezomib is a boronic acid pharmacophore-based proteasome inhibitor, originally approved for relapsed or refractory multiple myeloma (Figure 1.4.A). At the end of 2006, bortezomib was approved for use in mantle cell lymphoma patients who had received at least one prior therapy. This indication was extended in 2008 to include all stages of multiple myeloma, allowing for the use of bortezomib as a first line treatment. As of March 2010, over 160,000 patients have been treated with bortezomib. [196]

Bortezomib works in multiple myeloma by inhibiting proteasomal proteolysis. In primary MM cells, proteasome activity is inversely correlated to the apoptotic sensitivity of cells to proteasome inhibitors. [197] Interestingly, when looking at MM cell lines, this does not hold true. Altering the proteasome expression or proteasome workload of a cell lines changes its sensitivity to proteasome inhibition. [197] When one compares sensitive to resistant cells lines, sensitive cells express low levels of proteasomes and have higher levels of proteasome-mediated turnover. [197] While many researchers use peripheral blood mononuclear cells (PMBCs) as surrogate markers for proteasome inhibition in tumor cells, these results suggest that PMBCs may not accurately recapitulate the proteasome inhibition seen in tumor cells. [198]

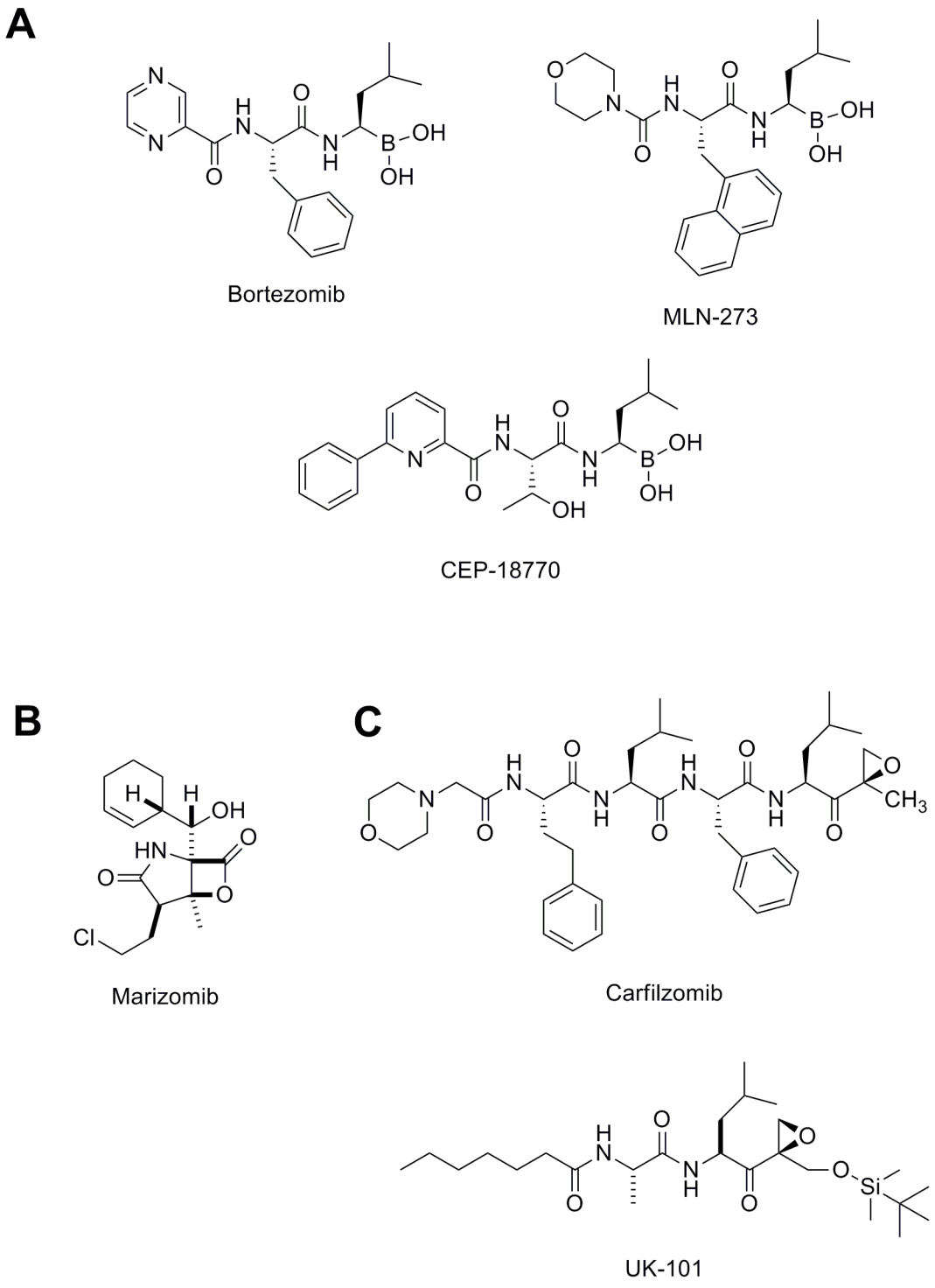


Figure 1.4: Proteasome inhibitors (circa 2008). The structures of published proteasome inhibitors are depicted. **A.** Boronic acid pharmacophore containing proteasome inhibitors. **B.** Marizomib, the beta-lactone proteasome inhibitor. **C.** Epoxyketone proteasome inhibitors, including our lead compound, UK-101.

Mechanistically, bortezomib targets β5, β5i, β1, and β1i at clinically relevant concentrations; as one might expect, the T-L activity is often increased after bortezomib treatment, as it tries to make up for loss of the other proteolytic activities. [198, 199] Treatment activates caspase-8- and caspase-9-dependent apoptosis. [199]

Due to the resounding success of bortezomib in MM patients, Millennium quickly began trials of bortezomib alone or in combination for other indications, mostly cancers. These studies have indicated that proteasome inhibitors in combination therapy will require optimization of treatment sequence and interval in addition to dose. [200] Preclinical evidence in other blood-borne cancers suggests that bortezomib also causes caspase-4-mediated (ER stress-induced) apoptosis. [201] Likewise, neoplastic B cell lines which overexpress β1i and β2 were most sensitive to bortezomib, while treatment of solid tumor cell lines with interferon-γ enhanced their sensitivity to bortezomib. [177] There have been 561 clinical trials of bortezomib, Phase I-IV, and 214 are currently open. [202]

While the efficacy of bortezomib was well-established in hematological malignancies, its potential as a monotherapy in solid tumors, especially those with limited therapeutic options and low survival rates, needed to be investigated. Preclinical studies in non-small cell lung cancer (NSCLC) examined mechanistically which characteristics lead to sensitivity to bortezomib treatment. They found that, to cause apoptosis, bortezomib must do two things: inhibit proteasomal proteolysis and overcome the cell's intrinsic resistance to apoptosis. [203] Using apoptosis as an end-point has been suggested to say as much about a cell's lack of general resistance mechanisms, such as efflux pumps or proteasome overexpression, as it does about its actual sensitivity to loss of proteasome function. [203] As Mortenson and colleagues suggest, "Therefore, the effect of bortezomib may not be sensitization of cancer cells to the apoptotic effect of chemotherapy, but may be modulating the cellular response to the chemotherapeutics and thereby accentuating cell death." [200] A study in ovarian cancer showed that cancerous cells have increased levels of proteasome expression and polyubiquitinated proteins compared to immortalized epithelial ovarian tissues, and bortezomib can cause p53-independent apoptosis in these cell lines. [204] The authors conclude the proteasome may be a good target in solid epithelial tumors: "In sum, elevated proliferation and metabolic rate resulting from malignant transformation of the epithelium stresses the [ubiquitin-proteasome pathway] and

renders ovarian carcinoma more sensitive to apoptosis in response to proteasomal inhibition." [204]

Combination studies with bortezomib are also being explored. In pancreatic cancer cell lines, treatment with bortezomib induced aggresome formation, a cytoprotective response which was not seen in immortalized human pancreatic epithelial cells or *in vivo* in mouse pancreatic epithelial cells. [205] The researchers found that combination therapy with HDAC inhibitors could disrupt aggresome formation, leading to greatly potentiated apoptotic effects in pancreatic cancer cells. [205] In prostate cancer patients, researchers used docetaxel and bortezomib to treat androgen independent prostate cancer. While this therapy was feasible, tolerable, and showed antitumor activity, it was no more beneficial than monotherapy. [206]

Overall, bortezomib has been very successful in the clinic, making a difference in the lives of patients. As a first-in-class drug, it has paved the way for the fury of proteasome inhibitors that have reached investigational new drug (IND) status in the last eight years. However, its clinical use is strictly limited by dose-dependent severe toxicities, most notably peripheral neuropathy. Other serious side effects include neutropenia and thrombocytopenia, as well as gastrointestinal, heart, lung, and liver problems. [196] Therefore, further development of INDs in this drug class require painstaking safety and efficacy testing in Phase I trials to quantify and minimize these side effects.

The next proteasome inhibitor to be developed for the clinic came right on the heels of bortezomib. In 2003, new life was breathed into the β-lactone class of proteasomes inhibitors with the discovery and characterization of the natural product proteasome inhibitor NPI-0052/Salinosporamide A (now called marizomib) (Figure 1.4.B). [207] It was shown to inhibit the proteasome faster than bortezomib and its inhibition lasted longer as well. [201] Mechanistically, it appears to cause caspase-4-mediated apoptosis (induced by ER stress) in CLL cells, even with only a 15 minute drug exposure. [201] Xenograft mouse studies of human multiple myeloma cells showed reduced tumor size upon treatment with marizomib but no apparent toxicity. [208] Proteasome inhibition was prolonged in tumors and whole blood but not in other normal tissues. [208] Additionally, marizomib does not appear to be a substrate of the major ABC transporters involved in multidrug resistance. [209] There are currently four open Phase I clinical trials of marizomib. [202]

At the same time, Millennium was busy designing better boronic acid proteasome inhibitors. Their next inhibitor was a bit of a surprise, as it was a dipeptidyl boronic acid designed to target parasitic proteasomes, called MLN-273 (Figure 1.4.A). [210] Work by Millennium and others attempted to take advantage of small structural differences between human proteasomes and those from infections organisms to develop a new way to treat these diseases. [210-212] MLN-273 preferentially targets *Plasmodium* species and *Mycobacterium tuberculosis* proteasomes over human proteasomes. [210, 211] In *Plasmodium*, ubiquitinated parasitic proteins accumulate and cell death occurs, even in species resistant to current therapies, suggesting MLN-273 could serve as a novel anti-malarial drug. [210] Studies of MLN-273 bound to the *M. tuberculosis* proteasome show a unique binding pocket when compared to human proteasomes; the *M. tuberculosis* proteasome is functionally and structurally most similar to the *T. acidophilum* proteasome. [211] Currently, there are no clinical trials of MLN-273 in the United States. [202]

Cephalon, Inc got into the act in 2008 with the publication of their orally available boronic acid proteasome inhibitor, CEP-18770 (Figure 1.4.A). It preferentially targets the CT-L activity of the proteasome, but also inhibits some serine proteases at high concentrations. [213] While it has a similar tissue distribution when compared to bortezomib, it is less cytotoxic to normal human epithelial cells, bone marrow progenitors, and bone marrow-derived stem cells. [214] Combination treatment of MM cell lines with CEP-18770 and bortezomib or melphalan gave synergistic activity and resulted in apoptotic cell death. [215] However, PBMCs were not affected, even at doses 10-fold above those needed to kill MM cell lines. [215] While CEP-18770 is orally bioavailable in rodents, oral doses are approximately one-third as efficient as intravenous. [213, 215] Nevertheless, in a MM xenograft model, CEP-18770 gives a higher complete response rate when compared to bortezomib while causing minimal changes in body mass. [214] Combination therapy in an *in vivo* model shows that CEP-18770 chemosensitizes tumors to bortezomib or melphalan therapy with little tumor progression, change in body weight, or difference in overall survival when compared to monotherapy. [215] There are currently three total clinical trials of CEP-18770 in the United States, 2 open trials and one completed trial. [202]

The next inhibitor developed for the clinic was PR-171 (now called carfilzomib), an epoxyketone registered by Proteolix, Inc (Figure 1.4.C). Carfilzomib works by binding primarily β5 and β5i and

inhibiting the CT-L activity of proteasomes in cell lines, patient primary tumor cells (MM, CLL, AML, NHL, and Waldenstrom's Macroglobulinemia (WM)), whole blood, and PBMCs. [216-218] Notably, carfilzomib inhibits other proteasome activities and subunits at higher concentrations; it may also be a substrate for permeability glycoprotein, a well characterized ABC transporter. [217] In MM cell lines, carfilzomib induces intrinsic and extrinsic apoptosis as a single agent while also acting synergistically with dexamethasone. [217] More interestingly, carfilzomib was still effective in some cell lines which were resistant to other therapeutics (bortezomib, dexamethasone, or melphalan) as well as in bortezomib-resistant primary cells. [217]

Later studies showed that more specific drugs, which block only β5 or β5i, were not cytotoxic in MM, PBMCs, leukemia, or lymphoma cell lines but that inhibiting both results in apoptosis. [92] In combination with bortezomib, carfilzomib was additive or synergistic in WM and B cell lymphoma (BCL) cell lines, depending on the dose used. [218] As a single agent, carfilzomib caused apoptosis in WM and BCL cell lines in caspase-dependent and –independent manners. [218] More recent studies suggest a role for carfilzomib in the treatment of lupus, as it prevents disease progression in a lupus-prone mouse model. [219]

While there are currently 11 clinical trials of PR-171 open, only one has been published. This Phase I study determined the maximum tolerated dose of carfilzomib in patients as 15mg/m^2 when treated for five consecutive days followed by nine days rest. [216] Carfilzomib achieved wide tissue distribution, but the elimination half-life was less than 30 minutes. [216] All patients reported side effects of therapy, most often Grade I/II gastrointestinal problems. [216] Of the 28 patients treated in this study, four were considered responsive (carfilzomib ≥ 11mg/m^2) and nine had stable disease. [216]

Another epoxyketone proteasome inhibitor was reported in 2007 by us, called UK-101 (Figure 1.4.C). [220] Since previous work had demonstrated that the P2 serine was not critical for the binding of dihydroeponemycin, derivation at the P1' and P2 positions of this molecule produced a library of inhibitors. [220, 221] UK-101 was selected due to its specificity for the β1i subunit of the immunoproteasome, as no immunoproteasome specific inhibitors had thus far been developed. In prostate cancer cell lines, UK-101 was able to induce apoptosis, but it did not inhibit angiogenic sprouting of fibroblasts, a process which relies on the constitutive proteasome. [220] Later computational modeling of UK-101 bound to β1i suggested the basis for its specificity was a steric clash between the P1' group and the binding pocket of β5. [222]

The work contained within this dissertation builds upon these studies to examine the importance of β1i for prostate cancer growth, using the PC-3 cell line as a model, and examines in more detail the binding preferences of the lead β1i inhibitor, UK-101.

Chapter 2: Specific Aims & Hypotheses

The overall goal of this research is to develop small molecule modulators to utilize as molecular probes of immunoproteasome function and to explore as therapeutic agents for cancer. Immunoproteasomes, alternatively composed proteasomes found constitutively in cells of hematopoietic origin but inducible in other cells types, are not functionally well understood. While their expression is modulated in a number of disease states, the functional consequences of these changes in protein levels is not clear. Some cancer cell lines upregulate the expression of the beta catalytic subunits of the immunoproteasome. [223] In solid tumors, an inflammatory microenvironment may induce immunoproteasome expression as well. Studies suggest that cells which express immunoproteasomes are sensitive to their inhibition, and thus, immunoproteasome inhibitors could be a viable therapeutic option for certain solid malignancies. However, no immunoproteasome-specific small molecules were available at the time this research began, so our ability to elucidate the functional importance of immunoproteasomes was limited, and inhibiting immunoproteasomes selectively was not possible.

The approval of the proteasome inhibitor bortezomib (Velcade®) by the FDA for the treatment of multiple myeloma and refractory mantle cell lymphoma validated the catalytic subunits of the proteasome as chemotherapeutic targets. However, the clinical utility of bortezomib was limited by severe systemic toxicity, including dose-limiting peripheral neuropathy, which was thought to be a class effect of proteasome inhibition. Therefore, the development of more specific and selective inhibitors gained popularity as a methodology to minimize this toxicity. Targeting the immunoproteasome was also an attractive strategy, as this proteasome subtype is not expressed in peripheral nerves and thus immunoproteasome inhibitors were expected to show milder side effect profiles than bortezomib. Such efforts led to the development of UK-101, a β1i-subunit specific inhibitor, in the lab of Dr. Kyung-Bo Kim [220, 223]. UK-101 has shown an ability to inhibit the proliferation of cancer cells expressing increased levels of β1i while having a minimal effect on “normal” cells.

The major purpose of the research described herein was to validate the mechanism of action of UK-101, proposed to be the inhibition of β1i, while simultaneously validating β1i as a chemotherapeutic target in the PC-3 prostate cancer cell line. Previous work showed that UK-

101 inhibited β1i *via* covalent modification, using multiple cancer cells lines, and caused apoptosis after 48 hours [223]. However, the effects of UK-101 on other cellular proteins were unknown. Our hypothesis was twofold. First, we hypothesized that the expression and catalytic activity of β1i is important for the growth and proliferation of the PC-3 cell line. Therefore, we hypothesized that the apoptotic effect seen upon treatment of PC-3 cells with UK-101 was due solely to its covalent inhibition of β1i.

Using orthogonal tools to modulate the relative abundance and activity of β1i, we attempted to validate that the anti-proliferative effects seen upon treatment with UK-101 were due to the covalent modification of β1i and not other off-target effects. In these same systems, we examined the effect of modulating the expression of β1i on PC-3 cellular proliferation, to determine the general feasibility of a β1i-targeting approach in cancer cell lines. The methodology utilized to achieve these goals is elucidated in the following aims:

Aim 1: Validate the target of UK-101 in the PC-3 prostate cancer cell line is β1i

Aim 1.1: Using natural inducers of immunoproteasome subunits, determine the relative sensitivity of PC-3 cells to UK-101.

Aim 1.2: Using RNAi-mediated knock-down of β1i protein levels, determine the relative sensitivity of PC-3 cells to UK-101.

Aim 1.3: Using biotinylated probes of the proteasome, examine the time- and concentration-dependent binding pattern of UK-101 in PC-3 cells.

Aim 2: Investigate the importance of β1i expression and function for proliferation in the PC-3 prostate cancer cell line

Aim 2.1: Using a siRNA-mediated knock-down of β1i protein levels, determine the effect of loss of β1i on the viability of PC-3 cells.

Aim 2.2: Using a plasmid-mediated overexpression of β1i protein levels, determine the effect of β1i and catalytically inactive β1i on the viability of PC-3 cells.

Chapter 3: Materials and Methods

A. Cell culture

The PC-3 prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in F-12K medium with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) or 10% Tet system approved fetal bovine serum (Clontech Laboratories Inc., Mountain View, CA) in tetracycline repressor transfected cell lines. Antibiotics were used as part of selection media only, as noted in **3S**.

B. Compounds

UK-101 was synthesized following procedures previously reported [220]. Human recombinant Tumor Necrosis Factor-α (TNF-α) from *Escherichia coli* (*E. coli*) was purchased from Sigma Aldrich (St. Louis, MO). Interferon-gamma was purchased from eBioscience (San Diego, CA). Bortezomib was obtained from ChemieTek (Indianapolis, IN). Epoxomicin, eponemycin, epoxomicin-biotin, eponemycin-biotin, carfilzomib, and YU102 were synthesized as previously described [191, 194, 221, 224, 225]. Puromycin dihydrochloride was purchased from Sigma Aldrich, G418 sulfate was purchased from Enzo Life Sciences Inc. (Farmingdale, NY), and Zeocin was purchased from Invitrogen (Carlsbad, CA).

C. MTS cell viability assay

PC-3 cells were plated at a density of 7,000-8,000 cells/well in a 96-well plate, and allowed at least 24 hours to attach. The indicated inhibitors were added in increasing concentration and cells were treated for 48 hours. The percentage of cell survival was determined using the MTS reagent, CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following the manufacturer's protocol. Briefly, 20µL of MTS reagent were added to cell samples in 100µl of culture media and incubated for one hour at 37°C. Absorbance was recorded at 490nm wavelength on a microplate reader (FL600; Bio-Tek Instruments, Inc., Winnoski, VT) using the software KC4 v.2.5 (Bio-Tek Instruments, Inc.). Cell proliferation was determined as a percentage relative to vehicle treated cells. IC_{50} values were calculated from sigmoid dose response curves by the method of nonlinear regression to a logarithmic function using GraphPad PRISM® (GraphPad Software, Inc, La Jolla, CA). These data represent the average of three or more replicates with error bars showing the standard error of the mean.

D. Caspase activity assay

Caspase 3/7, 8, and 9 activities were checked using the appropriate Caspase-Glo® kit for cell-based assays and following the manufacturer's instructions. Briefly, cells were plated in sterile white 96 well plates and treated in the same manner as they were for the MTS assay. At the end of the treatment time, 100µL of the room temperature assay dye was added to each well and mixed thoroughly (400 rpm for 30 seconds [Caspase 3/7] or two minutes [Caspase 8 & 9]). After an additional 30 minute incubation, luminescence was measured for each sample using a Veritas™ Microplate Luminometer (Promega Corp., Sunnyvale CA). Vehicle control was arbitrarily assigned a value of one and the fold increase of relative luminescent units was plotted in GraphPad PRISM® (GraphPad Software, Inc). These data represent the average of three or more replicates and error bars show the standard deviation.

E. Proteasome cell-based activity assay

PC-3 cells were plated in a white-walled 96-well plate at a density of 8,000 cells per well in 50µL of media per well. Cells were incubated at 37°C and 5% CO2 for 24 hours prior to treatment with either vehicle control, inhibitor control (10µM epoxomicin), or increasing concentrations (1µM, 10µM, and 50µM of UK-101; 5nM, 50nM, 500nM of epoxomicin or bortezomib) of proteasome inhibitors for two hours. Following equilibration to room temperature, 50µL of Proteasome-Glo™ Cell-Based Reagent (Promega) containing the luminogenic proteasome substrate (CT-L, C-L, or T-L) in the appropriate buffer conditions was added to each well, according to the manufacturer's instructions. Samples were mixed on a plate shaker per the manufacturer's instructions and incubated at room temperature for ten minutes. Luminescence readings were then recorded for each sample using a Veritas™ Microplate Luminometer (Promega Corp.). After subtracting the blank control, values were normalized to vehicle control and graphed in Microsoft® Excel™ (Microsoft, Redmond, WA). Values represent means and error bars display standard deviation.

F. Immunoblotting

Whole cell lysates were prepared by incubating cells in non-denaturing lysis buffer (50nM Tris-Cl, 150mM NaCl, 1% NP-40, 1% Triton X-100, and 1% protease inhibitor cocktail (Sigma-Aldrich)) on ice for one hour. Cells were then centrifuged at 14,000 rpm for ten min at 4°C (Sorvall Biofuge Primo R, Kendro Laboratory Products, Newtown, CT). Supernatants were collected and

subjected to protein assay *via* the method of Bradford using Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA). Protein concentrations were determined by a GENESYS 10 spectrophotometer and used to ensure equal protein loading (Thermo Spectronic, VWR, Arlington Heights, IL). Alternatively, cells were lysed in 1X Passive Lysis Buffer, according to the manufacturer's instructions (Promega). Equal volumes were used for blotting, *in lieu of* protein assay. All lysates were denatured by addition of 2x Laemmli Sample Buffer (Sigma Aldrich) and incubation at 100°C for ten minutes.

Subsequently, the denatured lysates were resolved by 12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% skim milk (Bio-Rad) or 5% bovine serum albumin (Sigma-Aldrich) in tris-buffered saline with 0.05% tween-20 (TBST) for one hour at room temperature with agitation. Appropriate primary antibodies were used to incubate the membranes overnight at 4^{o}C. [β1i, AbCam (ab78336); β-actin, Novis Biologicals (NB600-501); GAPDH, Santa Cruz (sc-47724); Ubiquitin, Santa Cruz (sc9133); Myc-Tag (9B11), Cell Signaling (2276); Streptavidin-HRP, Pierce (21126)] Secondary antibody incubation was done using agitation for one hour at room temperature in 3% blocking solution. Finally, Pierce ECL Western Blotting Detection Reagents (normal or femto) (Thermo Fisher Scientific, Rockford, IL) were used to visualize protein of interests on film (Thermo Fisher Scientific).

G. Interferon-gamma treatment

PC-3 cells were treated with 100U/mL of IFN-γ or vehicle for 24 hours, then washed and given fresh media. Cells were harvested at the indicated time points after removing the treatment media, lysed, and blotted as in **3F**. Alternatively, PC-3 cells were treated then subcultured into 96 well plates and treated as in **3C** for the MTS assay.

H. Tumor necrosis factor-alpha treatment

PC-3 cells were plated onto p100 dishes and treated continuously with vehicle or 20ng/mL TNF-α for 72 hours and then subcultured. Cells were treated as in **3C** for the MTS cell viability assay. Cells were also collected from well plates every 24 hours and used for immunoblotting, as in **3F**.

I. Immunofluorescence

Coverslips were sterilized with ethanol and UV light in a 35mm dish. For the interferon-gamma conditions, cells were plated on the dish after pretreatment as indicated. At the end of the

experiment, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton-X in PBS. Coverslips were then blocked with 10% goat serum (Invitrogen) and 1% BSA in phosphate buffered saline with 0.05% tween-20 (PBST). Primary antibody was added in DakoCytomation Antibody Dilutant with Background Reducing Components at 1:800 (Dako, Carpinteria, CA). Subsequently, secondary antibodies were added (AF488 (Invitrogen) at 1:1000 and Rhodamin Phallodin (Invitrogen) at 1:1000) in the same antibody dilutant. Coverslips were washed well in PBST, mounted onto slides with Prolong Gold antifade reagent (Invitrogen), and allowed to solidify overnight. The next day, the coverslips were sealed and visualized using an inverted fluorescence microscope (Nikon Ti-U microscope) with NIS Element Research image analysis software (Nikon, Melville, NY).

J. siRNA

The cells were transfected according to the manufacturer's instructions using DharmaFECT2® siRNA transfection reagent (Thermo Fisher Scientific, Lafayette, CO). ON-TARGET*plus*® siRNAs were used. The positive control pool was for human GAPD [D-001830-10], the negative control pool was human non-targeting [D-001810-10], and the test pool was for PSMB9 (β1i) [L-006023-00-0005]. Additionally, the four individual oligos were tested from the β1i targeting pool [LU-006023-00-0002].

Briefly, PC-3 cells were plated at a density of 20,000 cells per well in a 24 well plate and allowed 24 hours to attach. The siRNA was diluted separately from the DharmaFECT2® and after a five minute incubation at room temperature the two were combined. They were then allowed to incubate for an additional 20 minutes prior to the addition of complete media. The final solution added to the cells contained 100nM siRNA (or 125nM, where indicated) and 0.5µL of DharmaFECT2® per well. 24 hours post-transfection, the transfection media was removed and fresh media was added. Media was refreshed every 24 hours thereafter and cells were subcultured when the untreated and mock transfected controls reached >70% confluence. Samples were collected at the time points indicated and subjected to western blotting analysis as detailed in **3F**.

K. Phase contrast microscopy

siRNA treated cells were grown (as above) in well plates and observed every 24 hours prior to the media change. The cells were visualized using an inverted microscope (Nikon Ti-U

microscope) with NIS Element Research image analysis software (Nikon).

L. Cell counting

After trypsinization, each well of cells was resuspended in an equal volume of media. An aliquot was taken and mixed one to one with trypan blue. 10μL of this cell solution was then added to a TC10 dual-chamber counting slides, in duplicate, and counted on a TC10™ Automated Cell Counter (Bio-Rad). The number of live cells per milliliter was used to determine the total live cell population from each well, and this was plotted using GraphPad PRISM® (GraphPad Software, Inc). Bars represent means and error bars show standard deviations.

M. Site-directed mutagenesis

Mutagenesis was performed using the Stratagene QuikChange® Site-Directed Mutagenesis Kit following the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). Briefly, polymerase chain reaction was performed with 5ng of DNA for 18 cycles of denaturation for 30 seconds at 95°C, annealing for 60 seconds at 55°C, and elongation for 300 seconds at 68°C. 125ng of primer was used of the following sequence: forward 3'-GAAGTCCACACCGGG**G**CCACCATCATGGCAGTGG-5' and reverse 3'-CCACTGCCATGATGGTGG**C**CCCGGTGTGGACTTC-5'. After Dpn I digestion, the plasmid DNA was transformed into One Shot® TOP10 Competent Cells (Invitrogen) according to the manufacturer's instructions and plated on agar. After miniprepping (**3N**) the mutation was confirmed by sequencing (Eurofins MWG|Operon, Huntsville, Alabama).

N. Plasmid DNA preparation (Miniprep)

Single colonies from agar plates were picked and incubated overnight in LB media with shaking. After making glycerol stocks, the media was centrifuged to pellet the bacteria. Using a 5Prime FastPlasmid™ Mini Kit (Gaithersburg, MD), the cells were lysed and transferred onto spin columns. After washing, the DNA was eluted; the concentration and purity were checked via a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

O. Subcloning

To transfer the β1i gene between plasmids, the enzymes Not I and Kpn I were used to digest the plasmid DNA for two hours at 37°C (New England Biolabs, Inc., Ipswich, MA). To transfer the 5'

end of the gene only, which contained the mutated base, Kpn I and Pst I digestion was performed for two hours at 37°C (New England Biolabs, Inc). The digested DNA was purified with either the QIAquick PCR purification kit or by running an agarose gel and using the QIAEX II gel extraction kit, as detailed in **3P**. Ligation was performed using the Quick Ligation Kit according to the manufacturer's instructions (New England Biolabs, Inc.). Ligated plasmids were transformed into competent cells and plated on agar, as in **3M**, and then miniprepped, as in **3N**. To confirm insertion of the gene prior to sequencing, the miniprep DNA was digested as before, run on an agarose gel, and stained with ethidium bromide to visual the DNA insert. All DNA plasmids positive for the insert were sent for sequencing.

P. Digest purification

After digesting DNA without an insert, the QIAquick PCR purification kit was used to purify the DNA according to the manufacturer's instructions (QIAGEN, Inc., Valencia, CA). To purify all other DNA, digestion reactions were separated by agarose gel electrophoresis. The DNA was excised and weighed, then purified using the QIAEX II gel extraction kit following the manufacturer's instructions (QIAGEN, Inc).

Q. Plasmid DNA preparation (Maxiprep)

E. coli were grown from glycerol stocks of sequenced DNA as in **3N** and then transferred to large flasks containing 150mL of LB media to grow with shaking at 37°C overnight. In the morning, the cells were pelleted and either lysed directly or stored at -20°C until lysis. DNA was purified using the GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit according to the manufacturer's instructions (Sigma Aldrich). After purification, the DNA concentration and purity was determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). DNA was stored at -20°C until transfection.

R. Plasmid DNA transfection

PC-3 cells were plated in six well plates at a density of 200,000 cells per well and allowed to attach for 24 hours. For each well to be transfected, 3.75µg of DNA was combined with 3.75µL of Plus™ reagent in media, then this solution was mixed with 3.75µL of Lipofectamine™ LTX in media according to the manufacturer's instructions (Invitrogen). The plating media was removed and replaced with two milliliters of OptiMEM® media (Invitrogen). A 0.5mL final

volume of transfection solution was then added to each well, bringing the total volume up to 2.5mL. After 24 hours the transfection media was removed and the cells were subcultured into p100 dishes with fresh, normal media.

S. Antibiotic selection and clonal expansion

The cells in p100 dishes from **3R** were allowed to attach for 24 hours. At 48 hours post-transfection, antibiotic selection began. Every three days new media containing antibiotics was added to the dishes over a total of two weeks. Based on data from the parental PC-3 cell line, the following doses of antibiotics were used for selection: puromycin, 0.5μg/mL; G418, 0.5mg/mL; Zeocin, 0.4mg/mL. Cells were then plated into 96 well plates at ten cells per milliliter of media and grown in selection media for two weeks. Colonies resulting from a single cell were then subcultured into progressively larger areas under selective pressure. Individual clones were named and used for tests as described.

T. Colony Formation Assay

Transfected PC-3 cells were plated at low density in six well plates and allowed to attach for 24 hours. Cells were either selected with the appropriate antibiotics, or induced (or not) with doxycycline (1μg/mL) (Sigma Aldrich). Media was changed every three days, including fresh drugs. Once visible colonies were observed during media change, cells were washed with saline and fixed for ten minutes in ice cold methanol. Cells were then stained for ten minutes with a 0.5% (w/v) crystal violet solution in 25% (v/v) methanol in water. Destaining was achieved with water washing and plates were allowed to dry overnight. Images were obtained using a Xerox scanner (Xerox Corp., Norwalk, CT) with image refinement in Adobe Photoshop (San Jose, California).

U. General cell viability assay

PC-3 cells were plated at low density (1,000 cells/well) in a 96-well plate and allowed to attach for 24 hours. Cells were induced or not with doxycycline (1μg/mL) and at appropriate time points, the percentage of viable cells was determined using the MTS reagent, CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Induction media was refreshed every three days. Curves reflect the absorbance of

the dye at 490nM and represent the number of viable cells per well, averaged, with error bars representing the standard deviation.

Chapter 4: Results

A. Proteasome inhibitors: 2009 to the present

In the last three years, a number of new proteasome inhibitors have been developed and many new discoveries about older proteasome inhibitors have been published. Perhaps the most significant study in this period was performed by Onyx Pharmaceuticals, the developers of a number of epoxyketone-based proteasome inhibitors. They definitively addressed the issue of peripheral neuropathy as a side effect of bortezomib treatment: is it a class effect or a pharmacophore effect? They found that boronic acids inhibit a variety of serine proteases *in vitro* and *in vivo* (cathepsin G, cathepsin A, chymase, dipeptidyl peptidase II, HtrA2/Omi) at concentrations near those utilized for proteasome inhibition. [226] As they concluded in this paper, "Our data are consistent with a model in which bortezomib reduces neurite length by dual inhibition of the proteasome (resulting in oxidative and proteotoxic stress) and the neuronal prosurvival protease HtrA2/Omi." [226] This demonstrates that peripheral neuropathy, a side effect seen in approximately 30% of bortezomib patients, is an off-target effect of the boronic acid pharmacophore, not purely a target-mediated adverse drug reaction.

This study also found that aldehyde inhibitors and epoxyketones do not cause detectable inhibition of serine proteases. [226] However, another study found more evidence that vinyl sulfone proteasome inhibitors target cysteine proteases. [227] Kisslev's group found that changing the pharmacophore of proteasome inhibitors alone was sufficient to change their binding preferences. [227] This study and others also demonstrated that, regardless of the pharmacophore, inhibiting only the CT-L activity of proteasomes is not sufficient to cause an apoptotic response in cancer cell lines, especially MM. [227-229] Finally, a study in prostate cancer cell lines showed that proteasome inhibition stimulates an autophagic response which assists cells in clearing protein aggregates to alleviate proteolytic stress. [230]

A study of bortezomib in breast cancer showed that chronic bortezomib treatment reduces estrogen receptor-α levels in breast cancer cell lines. [231] Surprisingly, it uncovered a link between bortezomib treatment and estrogen receptor-α-dependent gene transcription, whereby bortezomib reduces RNA polymerase II occupancy and reduces the proliferative effects of estradiol. [231] Bortezomib was also suggested to prevent disease progression in lupus-prone mice. [219]

MLN9708, a boronic acid proteasome inhibitor prodrug which is converted to MLN2238 in aqueous solution, was the first new proteasome inhibitor reported in the last three years (Figure 4.1.A). [232] It is more highly reversible than bortezomib, dissociating from proteasomes six-fold faster, with greatly improved plasma exposure and tumor distribution in rodent models. [232] It can be formulated for oral, subcutaneous, and intravenous delivery, and is effective in a xenograft prostate cancer mouse model. [232] It preferentially inhibits the CT-L activity of proteasomes, although it also targets the C-L and T-L activities at higher concentrations. [233] Importantly, it is not seen to inhibit the neuronal prosurvival protease HtrA2/Omi, a major mediator of bortezomib's neuropathic side effects. [233] MLN9708 is also able to induce apoptosis in cell lines, primary cells, and a xenograft model of MM with little apparent toxicity to PBMCs or mice. [233] As a single agent, MLN9708 increases the survival of xenograft mice when compared to bortezomib, and it has synergistic effects in combination with lenalidomide, HDAC inhibitors, or dexamethasone. [233] There are currently eight clinical trials of MLN9708, all of which are open. [202]

The other important general proteasome inhibitor developed recently is ONX 0912 (PR-047), an epoxyketone with improved solubility, metabolic stability, and oral bioavailability when compared to carfilzomib (Figure 4.1.B). [234] Kinetically, administration resulted in rapid inactivation of the proteasome systemically, with activity recovering through new proteasome synthesis in non-blood tissues within 24-72 hours. [234] It promotes antitumor activity in animal models when provided orally at less than the maximum tolerated dose, reducing tumor size and improving survival in two mouse models of MM. [234, 235] ONX 0912 is synergistic in combination with bortezomib or a pan-HDAC inhibitor, while it has additive effects with dexamethasone or lenalidomide. [235] There is currently one open Phase I trial of ONX 0912. [202]

Recently, the development of immunoproteasome inhibitors has been fruitful. Three new immunoproteasome-selective inhibitors have been published in the last three years, although no immunoproteasome inhibitors are yet in clinical trials. [202] The first inhibitor is ONX 0914 (PR-957), a β5i selective inhibitor (Figure 4.1.B). In the MOLT-4 human leukemia cell line, ONX 0914 shows a 20- to 40-fold preference for β5i over β1i or β5. [236] Interestingly, selective β5i inhibition was not found to affect proteasome function, although it does block inflammatory cytokine production in PBMCs as well as T-cell activation and differentiation. [236] In mice, only

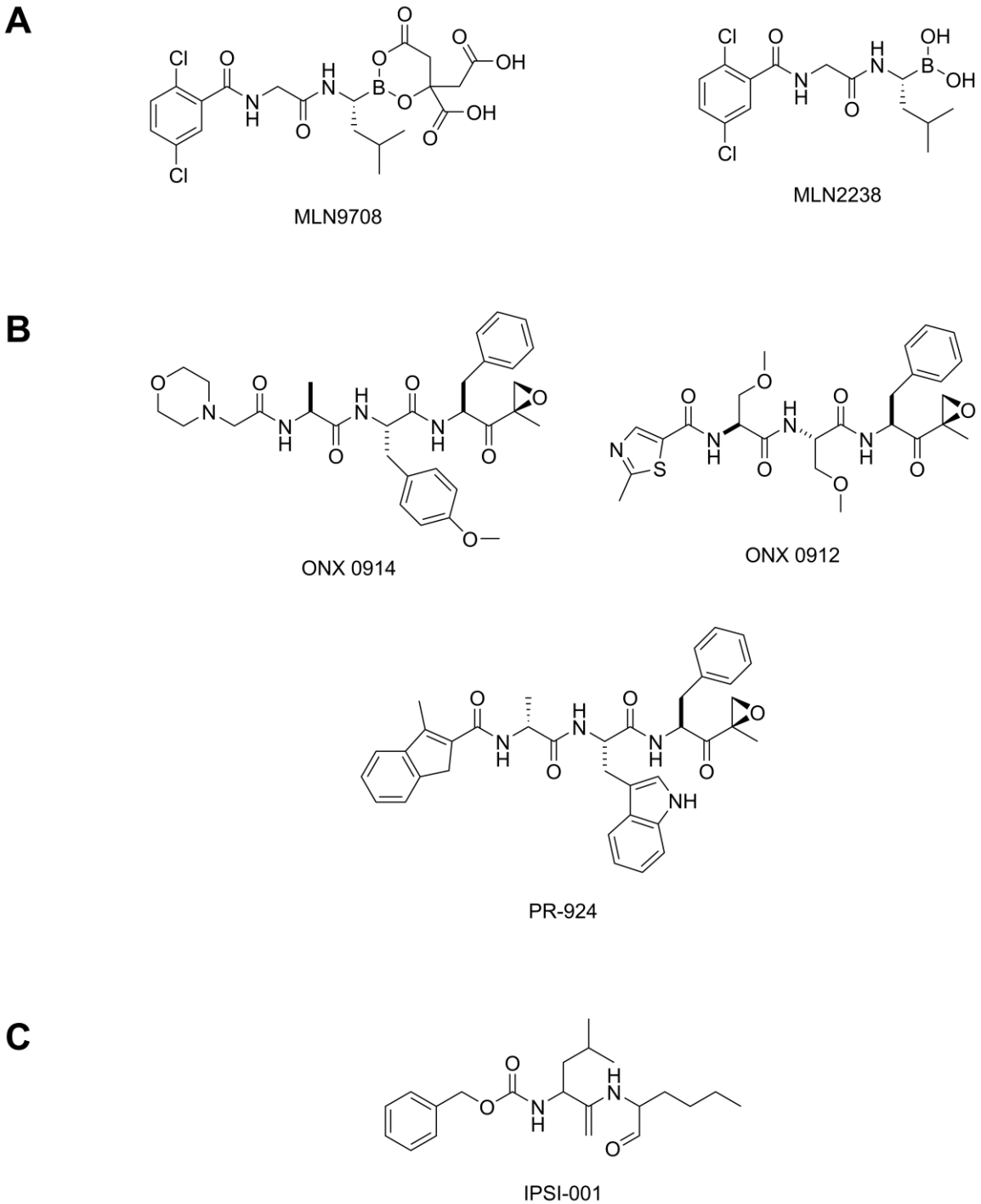


Figure 4.1: Proteasome inhibitors (circa 2011). The structures of published proteasome inhibitors are depicted. **A.** MLN9708, a prodrug which becomes MLN2238 in solution, is a new boronic acid pharmacophore containing proteasome inhibitor. **B.** Epoxyketone proteasome inhibitors, including the β5i-selective inhibitors, ONX 0914 and PR-924. **C.** An aldehyde-based β1i immunoproteasome inhibitor.

1-10mg/kg of ONX 0914 is necessary to inhibit β5i in the blood and kidney, with good tissue penetrance and an anti-inflammatory response at one-tenth of the maximum tolerated dose (30mg/kg). [236] ONX 0914 is also able to protect mice from dextran sulfate sodium-induced colitis, while a β5-selective inhibitor could not. [237] This suggests that β5i inhibition may be helpful for inflammatory diseases. Studies found ONX 0914 gave a similar therapeutic response as entranercept, an anti-tumor necrosis factor-α therapy, and bortezomib in animal arthritis and colitis models, respectively. [236, 237] Additionally, lupus-prone mice responded well to ONX 0914, showing lesser symptoms due to drug-mediated prevention of disease progression. [219]

Another immunoproteasome selective inhibitor developed in the last three years was PR-924/IPSI (Figure 4.1.B). PR-924 is 130-fold selective for β5i over β5, although it does also inhibit β1i in cells. [92] While β5i is inhibited by doses in the nanomolar range, the viability of tumor cells and PBMCs is not affected unless micromolar doses are used. [92, 238] MM cells treated *ex vivo* with PR-924 had a significant loss of viability, as did mouse xenograft models. [238]

The final immunoproteasome inhibitor reported thus far is IPSI-001, a peptide aldehyde which inhibits β1i and calpain (Figure 4.1.C). [239] PBMCs and MM cell lines with resistance to bortezomib are sensitive to IPSI-001, although human umbilical vein endothelial cells are not, suggesting an improved toxicity profile when compared to bortezomib. [239] Although IPSI-001 lacks the potency necessary for development as a therapeutic agent, it can be used to validate an immunoproteasome-targeting approach. [239]

B. Aim 1: Validate the target of UK-101 in the PC-3 prostate cancer cell line is β1i

Novel small molecules examined for efficacy as anticancer therapeutics are failing often in clinical trials. The reason for this attrition has changed significantly in the years between 1991 and 2000. In 1991, PK/bioavailability (40%), efficacy (30%), clinical safety, and toxicology (30% combined) were the top four reasons for attrition of all drugs in clinical trials. [240] By 2000, the problem with PK/bioavailability had declined to less than 10%, while problems with efficacy, clinical safety, and toxicology were as bad as or worse. [240] This strongly suggests that the methods by which scientists predict which molecules will have success in humans is quite flawed, resulting in huge expenditures on drugs which eventually fail. A more recent paper on Phase III and submission failure in 2007-2010 showed that two-thirds of drug failures at this stage were due to efficacy problems. [241] Additionally, the greatest number of drug failures

were due to anticancer drugs (n=23, 28%), and the author suggests that many of the oncology failures were due to assuming success in one tumor type would translate to other tumor types. [241] All of this points towards the necessity of additional preclinical work to define the mechanisms of action of new small molecules and to determine a compelling clinical rationale for their use in any particular type of cancer.

We have undertaken such studies during the development of our β1i inhibitor, UK-101. While UK-101 causes apoptosis in cancer cell lines, it is not yet clear whether this apoptotic effect is directly mediated by its irreversible inhibition of β1i. By choosing a sensitive cancer cell line and conducting all of our experiments within it, we examine the relationship between our drug and its target while minimizing other confounds. Previous work completed in our lab demonstrated that treatment of PC-3 cells with UK-101 led to apoptotic cell death at low micromolar concentrations. [220] Additional work showed that UK-101 modified β1i covalently in PC-3 cells, and that this covalent modification lasted at least 48 hours. [223] Thus, we chose the PC-3 prostate cancer cell line as our model system. Since off-target effects are major roadblocks for the development of new and effective pharmaceuticals, target validation studies in this system will assist in the further progression of β1i inhibitors towards preclinical trials. Our overall hypothesis is that the apoptosis seen upon treatment with UK-101 is due to the covalent modification of β1i.

We were interested in examining the sensitivity of the PC-3 cell line to proteasome inhibitors, including the new immunoproteasome selective inhibitors IPSI (PR-924) and PR-957 (ONX 0914). As shown in Figure 4.2.A, all seven inhibitors tested are capable of reducing the cell viability of PC-3 cells. The β5i-targeting selective inhibitors IPSI and PR-957 had IC_{50} values around 25μM, while UK-101 had an IC_{50} between one and five micromolar. This suggests that β1i may be more important for the viability of PC-3 cells. Alternatively, it could suggest that UK-101 is less subunit selective than IPSI and PR-957, as it does have a similar IC_{50} value as eponemycin, from which it is derived. Additionally, we wanted to examine the apoptotic pathway activated by treatment with UK-101. We used epoxomicin, the general epoxyketone proteasome inhibitor, as a positive control. After a 48 hour treatment in PC-3 cells, we found that both proteasome inhibitors activated all three caspase-types examined (Figure 4.2.B). This suggests that proteasome inhibition, whether by epoxomicin or UK-101, activates both the intrinsic and extrinsic apoptotic pathways.

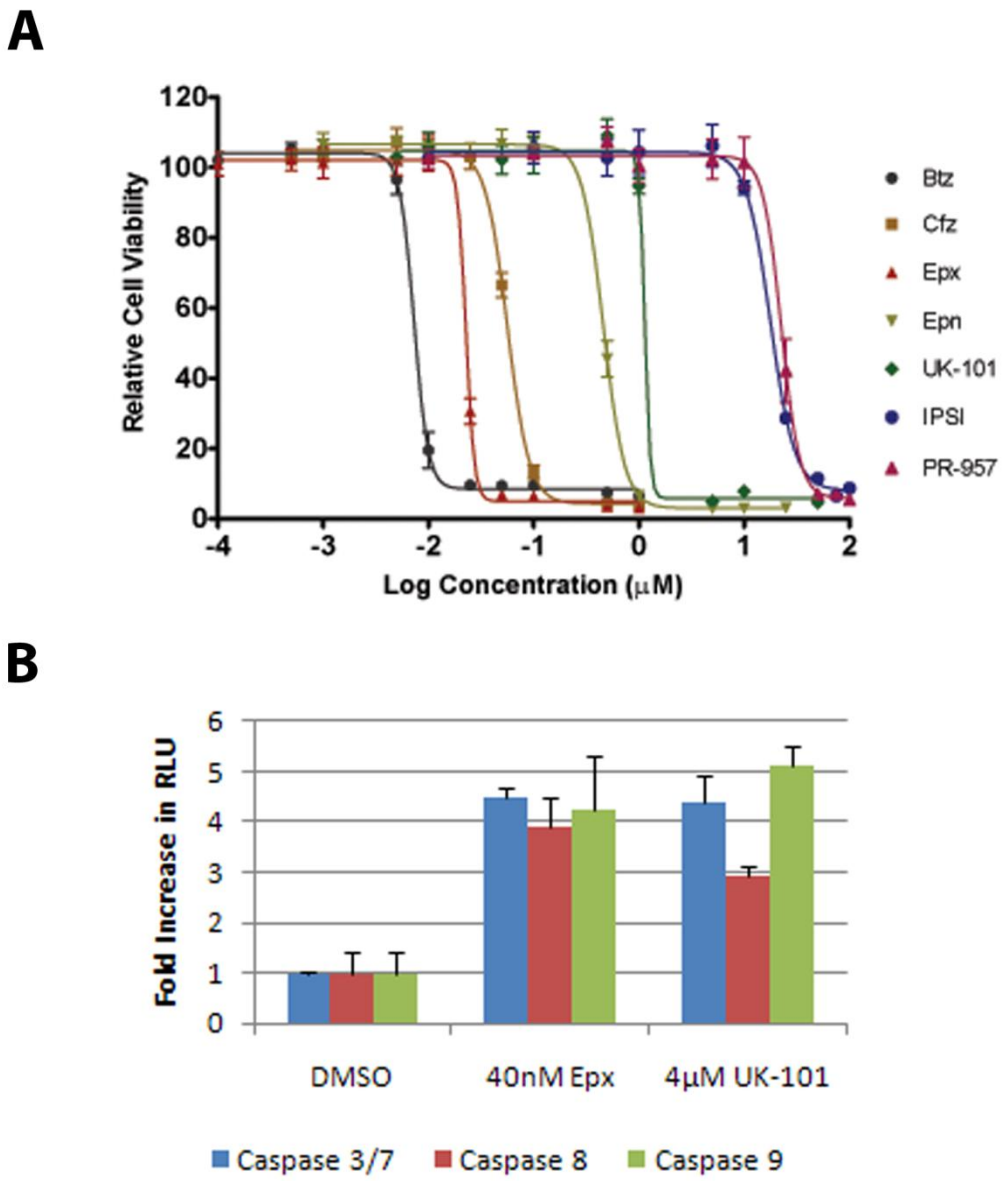
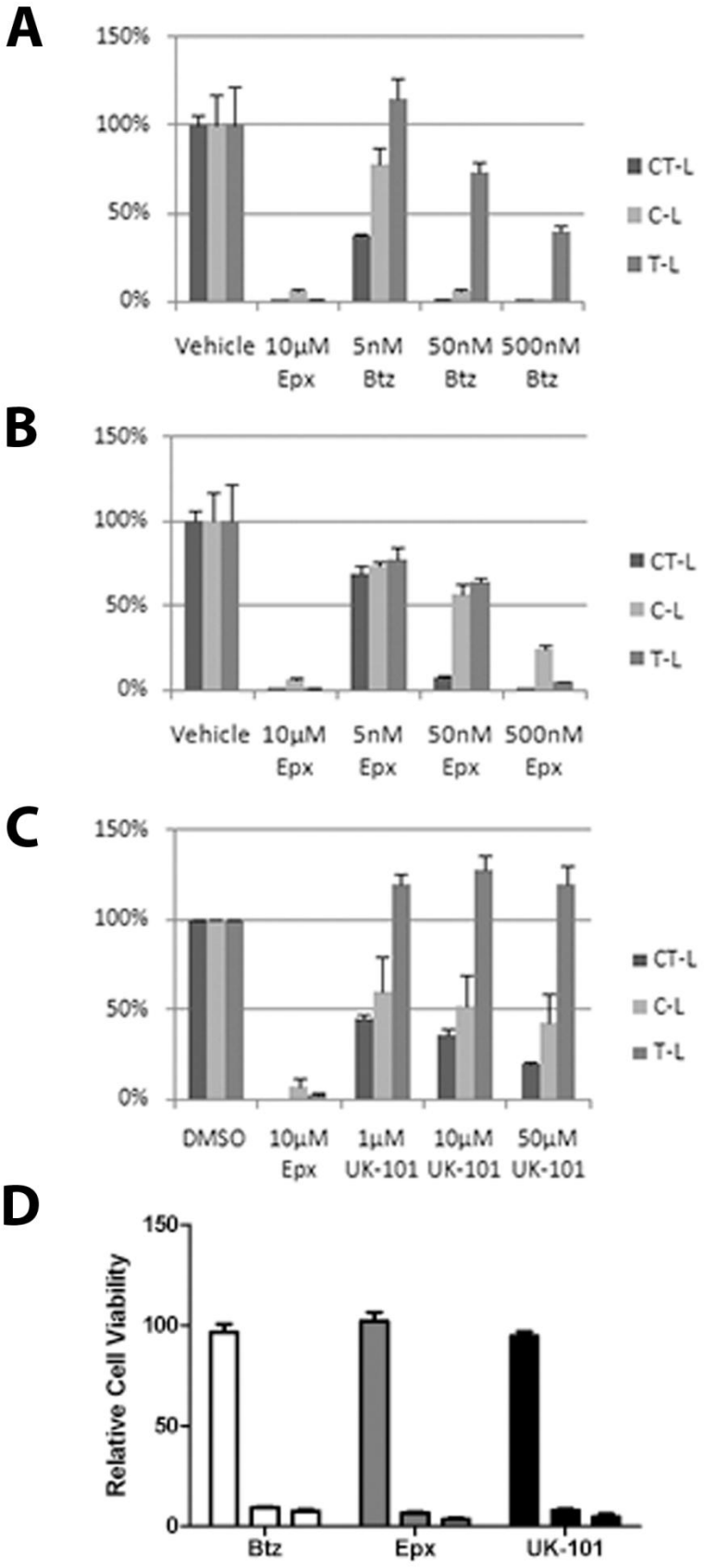


Figure 4.2: The effects of proteasome inhibition on the PC-3 prostate cancer cell lines. A. Cells were treated for 48 hours to determine viability. General proteasome inhibitors such as bortezomib (Btz), carfilzomib (Cfz), and epoxomicin (Epx) have low nanomolar IC_{50} concentrations. Eponemycin (Epn) has IC_{50} values in the high nanomolar range, while UK-101 has values in the low micromolar range. The new immunoproteasome specific inhibitors, IPSI and PR-957, also have IC_{50} values in the micromolar range, although higher than UK-101. **B.** Caspase activity assays in PC-3 cells after 48 hour incubation with inhibitors. Treatment of cells with concentrations slightly above the IC_{50} value for epoxomicin and UK-101 results in activation of all three caspase activities, confirming the loss of cell viability in **A** is due to apoptosis.

Previous work in the lab also showed that UK-101 could inhibit the CT-L activity of purified proteasomes. [223] We were interested to know whether this was also true for the proteasomes within the cells. Additionally, we wanted to explore the effect of UK-101 on the other typical proteasome activities, the C-L and T-L activity. Thus, we treated PC-3 cells with inhibitor for two hours and checked proteasome activity using a cell-based system, with high dose epoxomicin serving as a positive control. As expected, bortezomib preferentially targeted the CT-L activity in cells, although it inhibited the C-L and T-L activities at higher doses (Figure 4.3.A). Epoxomicin, on the other hand, targets all three activities, even at low doses, although higher doses demonstrate its preference for the CT-L and T-L activities (Figure 4.3.B). There has been some question as to which proteasome activities β1i is responsible for, as some have suggested it has C-L activity, similar to its homologue β1, while others have suggested it has primarily CT-L activity, based on its altered binding pocket. [21, 24, 25] In our cell line, low dose treatment with UK-101 reduced the CT-L and C-L activities while activating the T-L activity somewhat (Figure 4.3.C). This C-L activity inhibition does not change much as the dose increases, although the CT-L activity does decrease somewhat at the higher doses. If UK-101 is truly a β1i subunit selective inhibitor, this suggests that β1i is responsible for a significant amount of the CT-L and C-L activity in PC-3 cells, which would explain their sensitivity to treatment with this inhibitor. Surprisingly, when one compares the eventual reduction in cell viability caused by extending these two hour treatments over 48 hours, one finds the lowest concentration has little to no effect on cell viability, but the next highest concentration affects it significantly (Figure 4.3.D). In the bortezomib and epoxomicin treated cells, one can see a large change in the proteasome activity profile between these two treatments. However, there is little change in the proteasome activity profile for the UK-101-treated cells. The small change seen may be enough to induce apoptosis in this cell line. However, this may suggest additional targets for UK-101 which enhance its apoptotic effect.

C. Aim 1.1: Using natural inducers of immunoproteasome subunits, determine the relative sensitivity of PC-3 cells to UK-101.

Many cytokines, such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), are known to upregulate the levels of the immunoproteasome catalytic subunits, resulting in the cooperative expression of β5i, β1i, and β2i and their subsequent assembly into immunoproteasomes [242]. In the case of IFN-γ, this effect is achieved by a type II interferon


A
150%
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50μM UK-101
CT-L
C-L
T-L
D
Relative Cell Viability
150
100
50
0
Btz
Epx
UK-101

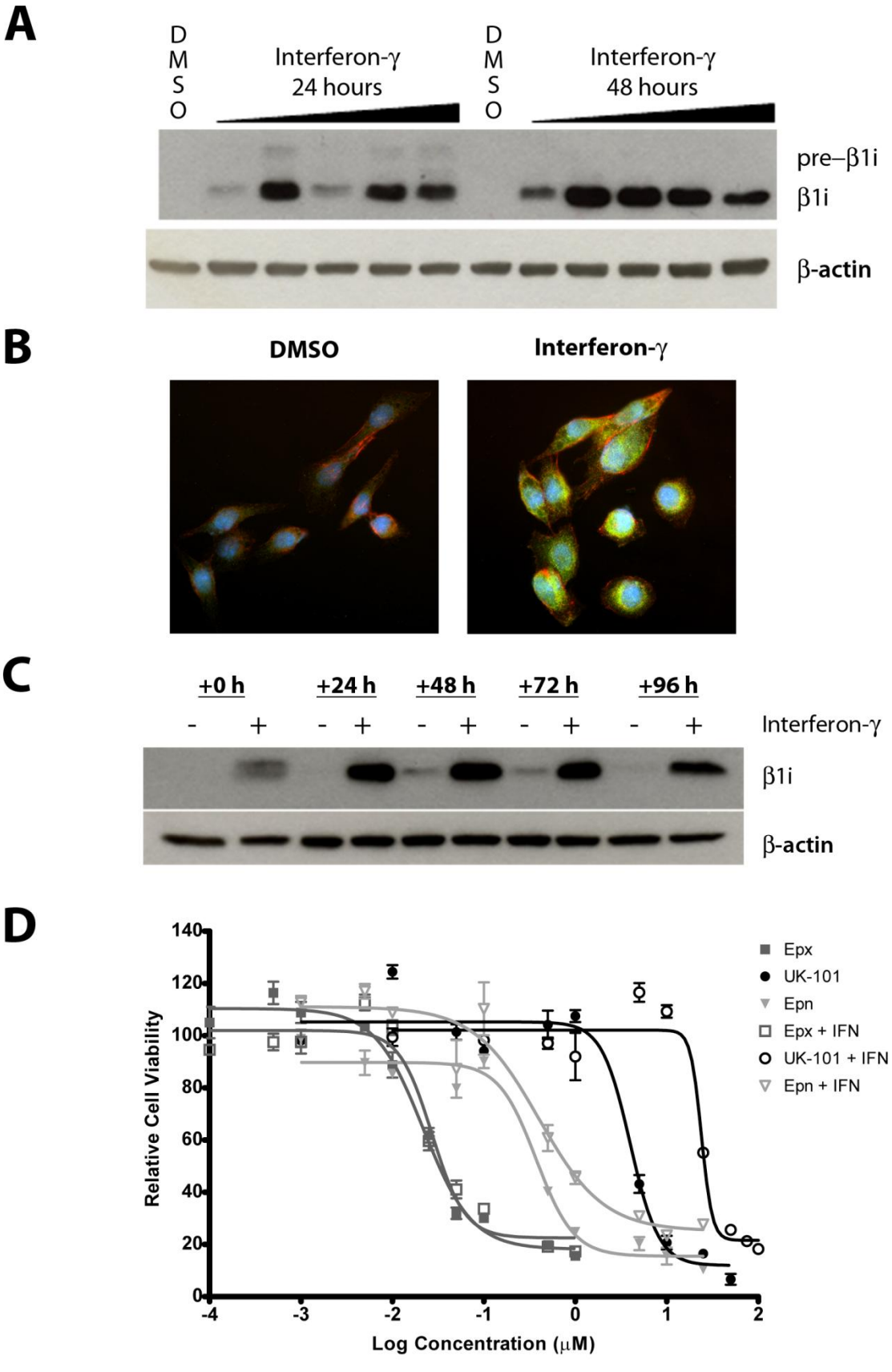
Figure 4.3: The effects of proteasome inhibition on proteasome activity in PC-3 cells. A-C. Cells were treated with proteasome inhibitors for two hours and then the CT-L, C-L, and T-L activity of proteasomes from the cells was measured. **A.** Bortezomib (Btz) treatment decreased all three proteasome activities at high doses, but preferentially inhibits the CT-L and C-L activities. **B.** Epoxomicin (Epx) treatment decreased all three proteasome activities, with little activity preference, although it is less effective at blocking the C-L activity. **C.** UK-101 decreases the CT-L and C-L activities, although it never blocks them completely. It also seems to activate the T-L activity. **D.** Cell viability of PC-3 cells treated with these same proteasome inhibitor concentrations over 48 hours. While partial inhibition of the proteasome is tolerable in these cells, more significant inhibition of more than one activity at two hours correlates with greatly reduced viability after 48 hours.

response utilizing interferon responsive element 1 (IFR1) [243]. For β1i, an additional factor, signal transducer and activator of transcription 1 (Stat1), binds to the promoter of β1i to upregulate β1i expression levels [244].

To determine whether IFN-γ could be utilized in this experimental paradigm, PC-3 cells were treated with increasing concentrations of IFN-γ over 24 or 48 hours and the expression of β1i was monitored via western blotting. As seen in Figure 4.4.A, a dose of 50U/mL of IFN-γ is sufficient to upregulate the expression of β1i. These results held whether the treatment time was 24 or 48 hours. Importantly, the upregulation of the subunit was shown to be predominately of the catalytically active form, suggesting that β1i is incorporated into proteasomes and processed to its catalytically active form within 24 hours of treatment. To confirm that this upregulation by IFN-γ did not alter the normal expression pattern of β1i, immunofluorescence was performed. As seen in Figure 4.4.B, even doses of 250U/mL did not appear to alter the expression pattern of β1i, but simply intensified the signal otherwise observed.

While these results were greatly encouraging, to perform target validation using the IFN-γ paradigm, the PC-3 cell expression of β1i would need to be upregulated for at least 72 hours. Thus, a time-dependent experiment was performed to examine the expression level of β1i after withdrawal of IFN-γ. A 24 hour pretreatment of 100U/mL of IFN-γ was chosen based upon the results above as it appeared to cause a significant upregulation of β1i at a minimal dose of IFN-γ. As seen in Figure 4.4.C, the upregulation of β1i was maintained for at least 96 hours after the removal of the IFN-γ from the system.

Using this overexpression system, a target validation experiment was performed to test the effect of IFN-γ-induced β1i overexpression on the sensitivity of PC-3 cells to UK-101. To determine whether this experimental paradigm affects the sensitivity to PC-3 cells to proteasome inhibitors in general, epoxomicin was used as a control. Additionally, eponemycin, which binds to proteasome subunits β1i, β5i, and β5, was included as an additional control. After pretreatment, cells were placed into 96 well plates and treated with proteasome inhibitors for 48 hours. Those cells pretreated with DMSO showed comparable IC_{50} values to those obtained previously [223]. The cells which had been pretreated with IFN-γ and treated with epoxomicin or eponemycin did not have any statistically significant changes in cell viability (Figure 4.4.D). However, those cells pretreated with IFN-γ and then treated with UK-101


A
DMSO
Interferon-γ 24 hours
DMSO
Interferon-γ 48 hours
pre–β1i
β1i
β-actin
B
DMSO
Interferon-γ
C
+0 h
+24 h
+48 h
+72 h
+96 h
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UK-101 + IFN
Epn + IFN

Figure 4.4: Interferon-γ-induced upregulation of β1i affects the sensitivity of PC-3 cells to UK-101. A. Treatment of PC-3 cells with increasing concentrations (50 units(U)/mL to 250U/mL) of IFN-γ for 24 or 48 hours induces a large increase in the expression of β1i. This β1i is found to be predominately in the catalytically active form. **B.** The upregulation of β1i expression seen *via* western blot is also apparent using immunofluorescence. There appears to be no change in β1i localization upon induction, or any variation in cellular morphology, even with this high dose (250U/ml) of IFN-γ. **C.** Pretreatment with IFN-γ causes a sustained upregulation of β1i protein levels, lasting at least 96 hours after IFN-γ is removed from the media. **D.** Based on the information in **C**, cells were pretreated with IFN-γ and subcultured in to 96 well plates for the MTS cell viability assay. A large right hand curve shift is seen in PC-3 cells pretreated with IFN-γ and exposed to UK-101, which represents a six-fold increase in IC_{50} value (4μM to 24μM). The change seen in the eponemycin treatment group fell within the 95% confidence interval of the nonlinear regression curve fit and thus was not deemed significant.

showed a six-fold increase in IC_{50} value (Figure 4.4.D). This suggests that an increase in the amount of β1i in PC-3 cells significantly impacts the ability of UK-101 to decrease the viability of these cells. This result supports the hypothesis that UK-101 causes apoptosis in PC-3 cells via its covalent modification of β1i.

Likewise, TNF-α is also capable of upregulating the expression of β1i, although not as strongly as IFN-γ. A 72 hour pretreatment was required to sustain β1i upregulation after withdrawal of TNF-α; expression of β1i decreases over time, nearing basal expression after 72 hours post-withdrawal (Figure 4.5.A). When cells pretreated with TNF-α were then exposed to proteasome inhibitors, the IC_{50} values did not change significantly. However, there were some notable effects at certain concentrations of bortezomib and UK-101 (Figure 4.5.B). TNF-α treatment appeared to protect bortezomib treated cells from loss of viability, which may be related to one of its mechanisms of action, proposed to be inhibition of NF-κB. [245, 246] Previous work showed that TNF-α can induce NF-κB activation in the PC-3 cell line. [223, 247-249] However, the opposite effect is seen in the UK-101 treated cells, where the cells are more sensitive to UK-101 after pretreatment with TNF-α (Figure 4.5.B). This is the opposite of the result we would expect if the sensitivity to UK-101 was due solely to the expression of β1i. However, both TNF-α and IFN-γ upregulate other cellular pathways and processes in addition to those of the immunoproteasome, so further experiments are necessary to definitively address our hypothesis.

D. Aim 1.2: Using a knock-down of β1i protein levels, determine the relative sensitivity of PC-3 cells to UK-101.

siRNA has quickly become a popular technique for exploring the effects of removing proteins from cells without many of the issues of temporal control associated with DNA knockouts. Since the completion of the human genome, siRNA molecules can be designed to knock down almost any protein. The first group reporting a β1i knock-down, Wang *et al,* achieved a 70% mRNA knock-down and a 65% protein knock-down using vector-based siRNA [250]. siRNA against β1i is commercially available, and one company had a guaranteed knock-down pool. A complete knock-down of β1i would allow for the determination of the relative proportion of the antiproliferative effects of UK-101 which do not derive from its binding to β1i.

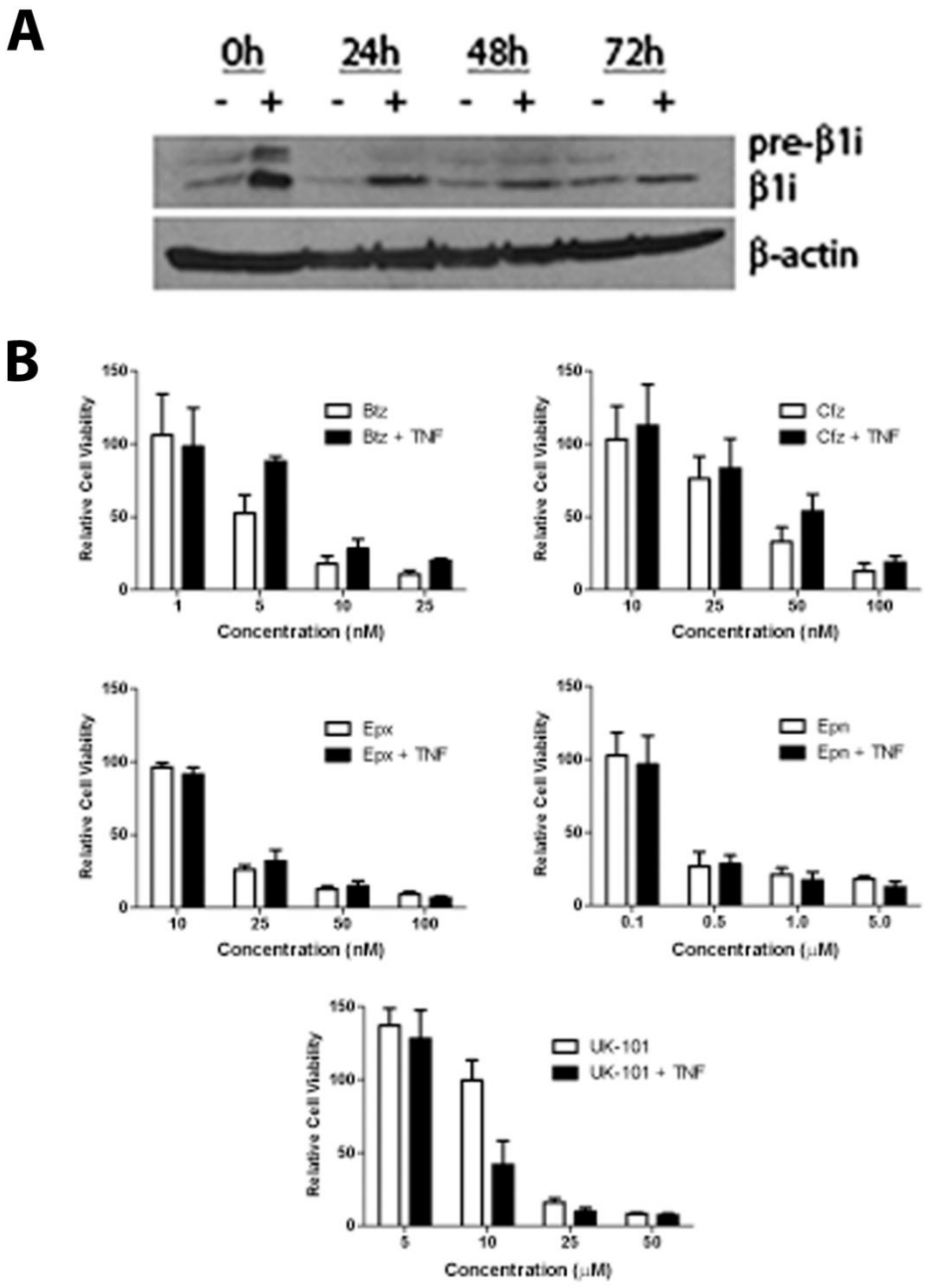


Figure 4.5: Tumor Necrosis Factor-α-induced upregulation of β1i affects the sensitivity of PC-3 cells to UK-101. A. Treatment of PC-3 cells with 20ng/mL of TNF-α for 72 hours induces a small increase in the expression of β1i. This β1i is predominately the catalytically active form and reduces relatively quickly when TNF-α is removed from the media. **B.** TNF-α treatment has a small affect on the sensitivity of PC-3 cells to proteasome inhibitors. Bortezomib seems to be less effective at 5nM in the TNF-α-treated cells while UK-101 is more effective at 10μM in the TNF-α-treated cells. However, there is no net change in IC_{50} value under these conditions.

We first set out to determine the degree and length of β1i knockdown using pooled siRNA. We found, similar to a GAPDH positive control pool, reduction in the expression of unincorporated pre-β1i after 24 hours, suggesting a reduction in newly synthesized β1i protein (Figure 4.6). As seen with the positive control, β1i siRNA decreased catalytically active β1i protein levels at 48 hours post-transfection, with significant knockdown occurring at 72 hours post-transfection. This reduction in β1i protein level was maintained up to 168 hours post-transfection. The scrambled control siRNA pool, which served as a negative control, had no effect on the expression of β1i or GAPDH. While the β1i protein level was undetectable by western blotting, some residual protein may have still been expressed in the cells. Nevertheless, this level of knockdown should be sufficient to see some effect on the sensitivity of transfected cells to UK-101.

To test this, we transfected cells with the scrambled control or β1i siRNA pools and tested their sensitivity to the proteasome inhibitors epoxomicin, eponemycin, and UK-101. The cells were treated with inhibitor at 96 to 144 hours post-transfection. As shown in Figure 4.7.A, there was no change in the sensitivity of the transfected cells to any of the inhibitors when comparing the scrambled control to β1i siRNA groups. This suggests that the effect of UK-101 on cell viability is not mediated by β1i. However, western blotting is a non-quantitative technique, so while the protein levels were undetectable using our β1i antibody, perhaps the knockdown did not reduce the protein levels sufficiently to see an effect. Thus, we transfected the cells using 125nM of siRNA (instead of 100nM) to look for a relationship between siRNA concentration and sensitivity to proteasome inhibitors. However, when we treated these cells we also saw no change in sensitivity to any of our proteasome inhibitors (Figure 4.7.B). This once again suggests an alternative target for UK-101 which is capable of inducing its antiproliferative effect.

To confirm this result, we transfected the PC-3 cell line with plasmid DNA containing a small hairpin RNA (shRNA) against β1i and selected for cells which were puromycin resistant. After two weeks, we obtained pooled knockdown clones which were clonally expanded under selective pressure from a single cell to create stable cell lines. As shown in Figure 4.8.A, we tested the knockdown efficacy compared to empty vector transfected cells and selected four clones of each type to move forward for additional testing. The knockdown efficacy of each clone differs, thus we expected to see differing effects on the sensitivity of the knockdown clones to UK-101. However, there was almost no change in the viability of the cells between

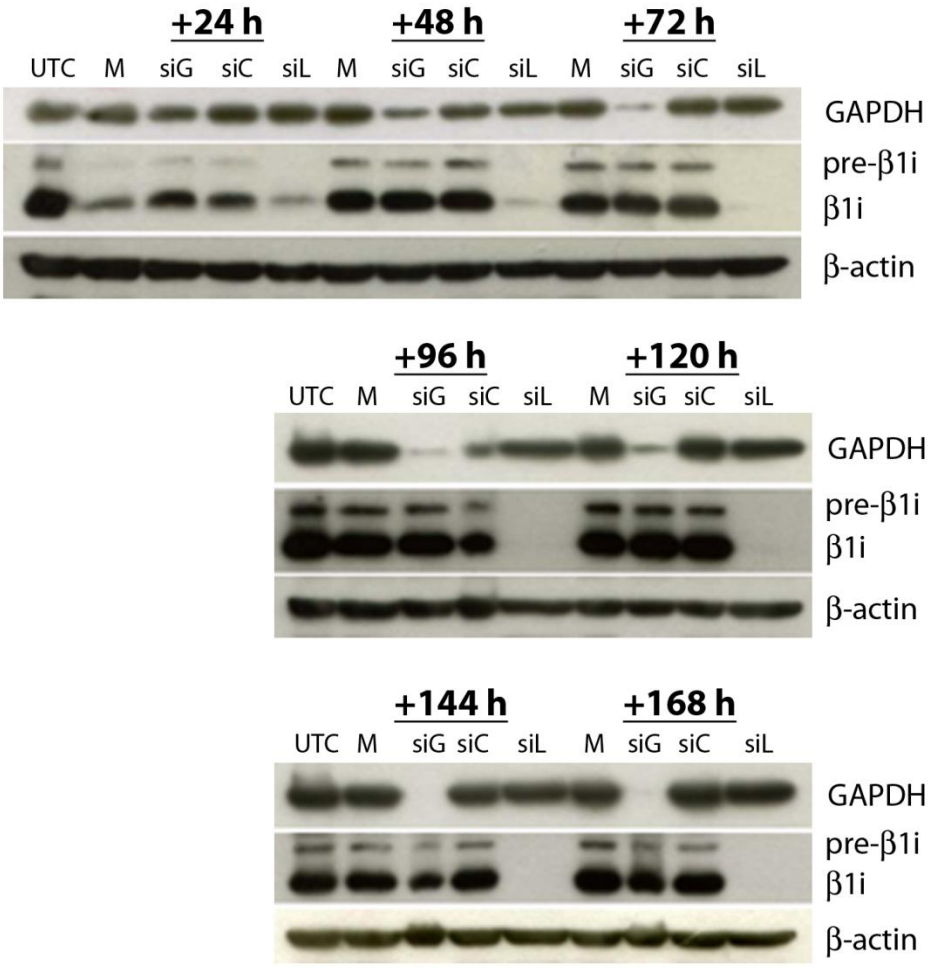


Figure 4.6: siRNA-mediated knockdown of β1i results in loss of protein expression. Western blotting shows that siRNA-mediated knockdown of the control protein GAPDH and the experimental protein β1i begins at 48 hours post-transfection and can be maintained for up to 168 hours post-transfection. [UTC = untreated control; M = mock transfected; siG = siRNA against GAPDH; siC = siRNA scrambled control; siβ = siRNA against β1i]

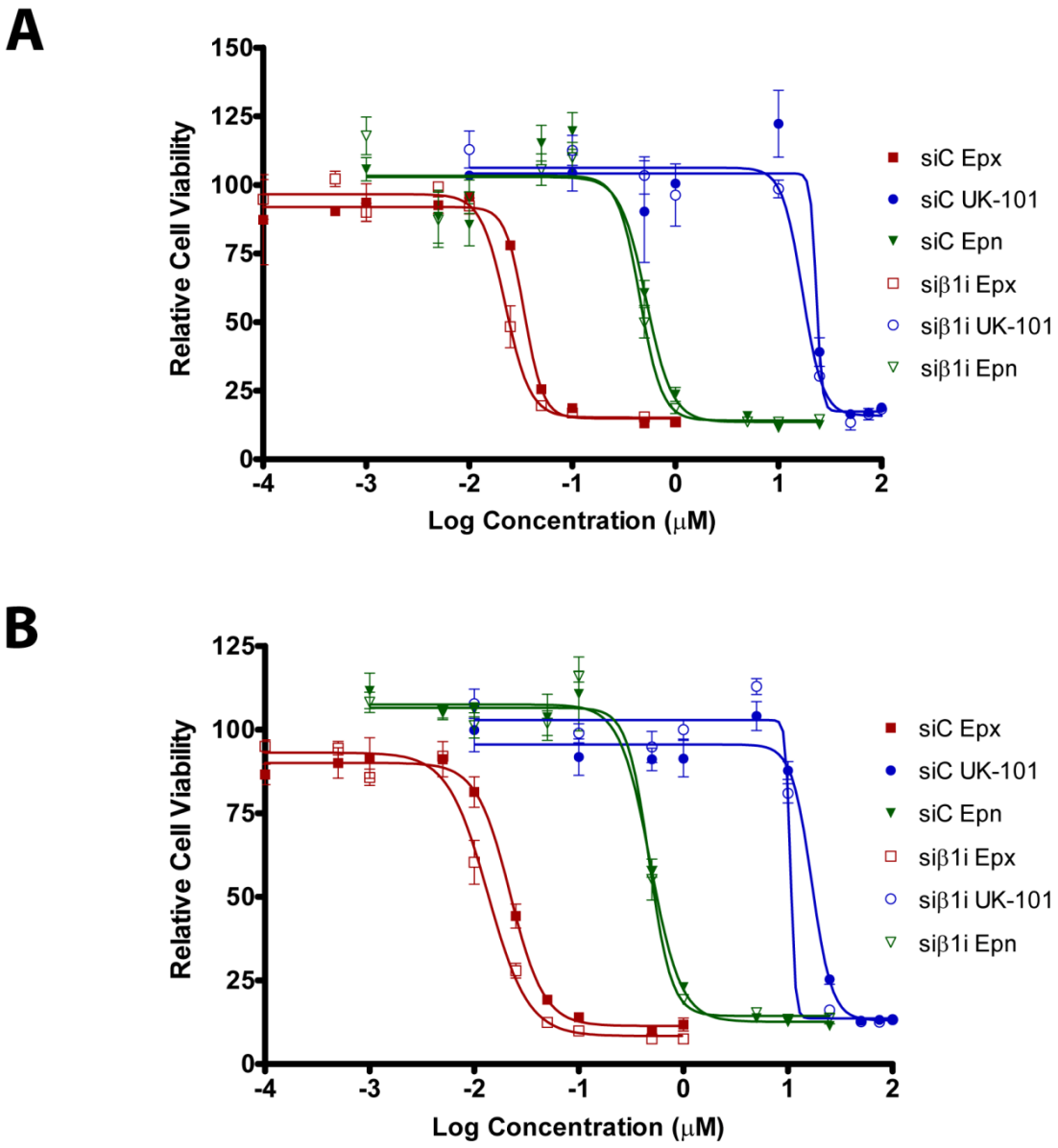


Figure 4.7: siRNA-mediated knockdown of β1i does not affect the sensitivity of cells to proteasome inhibition. MTS cell viability assay between 96 and 144 hours post-transfection. **A.** MTS assay from cells treated with 100nM siRNA shows no significant difference between scrambled control siRNA and β1i siRNA. **B.** MTS assay results obtained from cells treated with 125nM of siRNA show the same results as in **A**.

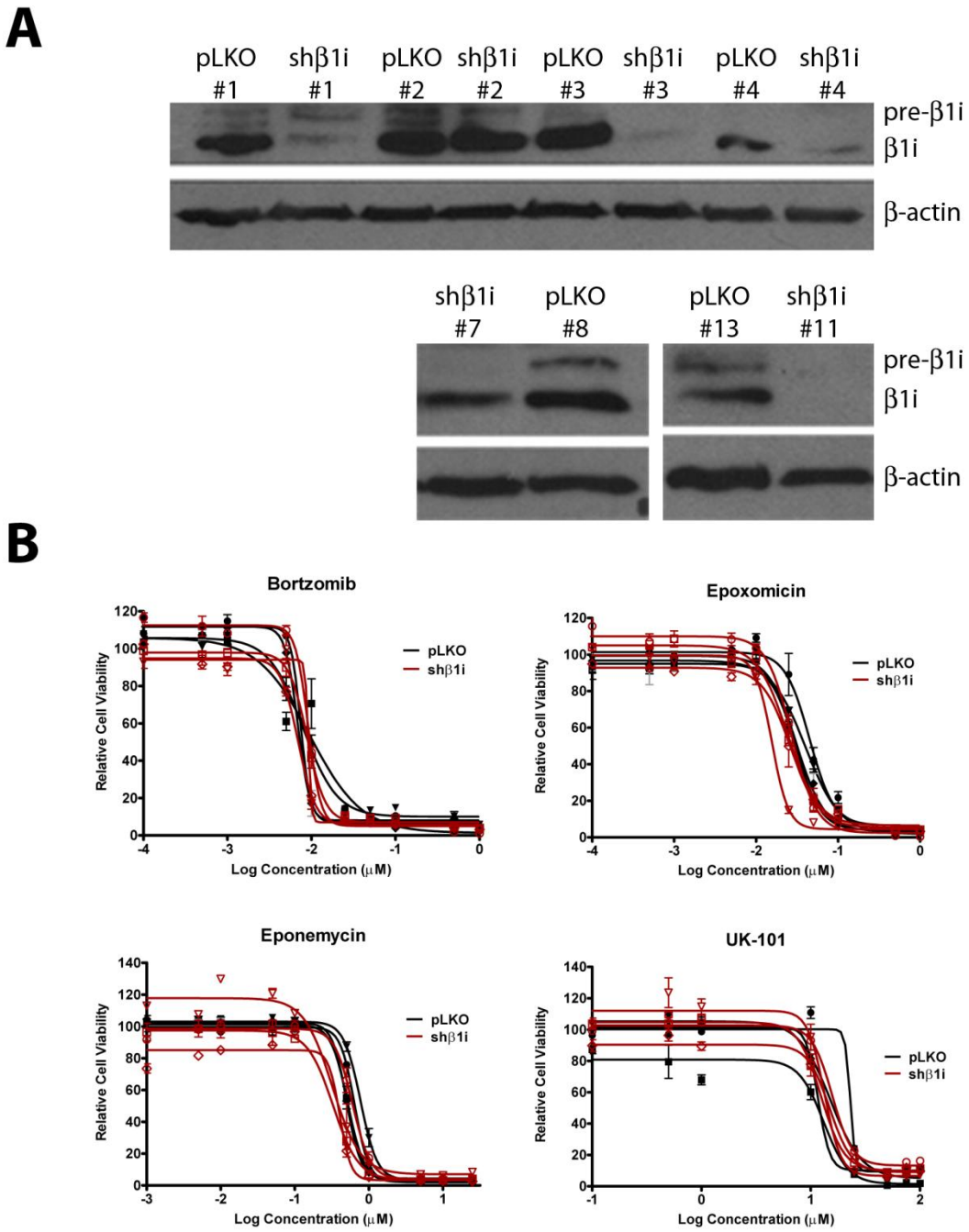


Figure 4.8: shRNA-mediated knockdown of β1i does not affect the sensitivity of cells to proteasome inhibition. A. Western blot of β1i expression in single cell clones containing either the shRNA targeting β1i or the empty vector. Expression of β1i is reduced to varying degrees in the shβ1i clones. **B.** MTS assay results obtained from selected clones shows no effect of the plasmid DNA (black lines, pLKO empty vector; red lines, shβ1i in pLKO vector) on the sensitivity of cells to UK-101 or other proteasome inhibitors.

clones (Figure 4.8.B) for any of the proteasome inhibitors examined, including UK-101. This result, akin to the siRNA data, does not support our hypothesis that UK-101 mediates its apoptotic effect through the inhibition of β1i.

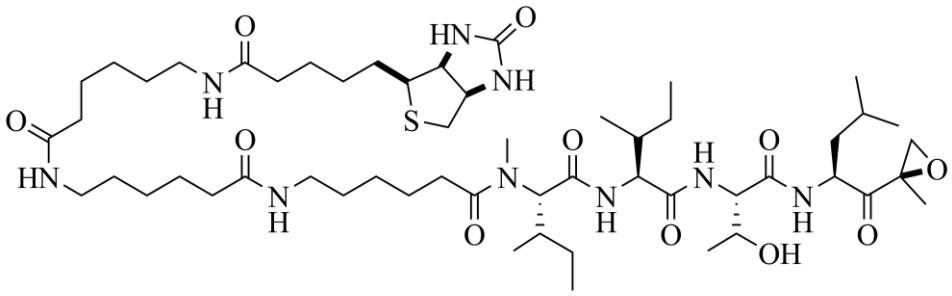
However, a major drawback of a RNA knockdown approach is the existence of a homologous protein (β1) which can be substituted in the assembly process for the missing β1i, as β1i serves as a scaffold protein for the formation of the immunoproteasome complex. As small molecule inhibitors of β1i do not affect the assembly of proteasomes, but siRNA and shRNA do, knocking down the protein will not have the same effect as simply inhibiting its catalytic activity. Still, the sensitivity of PC-3 cells to UK-101 regardless of the expression level of β1i strongly suggests that the antiproliferative effect mediated by UK-101 is not due solely to its covalent modification of β1i.

E. Aim 1.3: Using biotinylated probes of the proteasome, examine the time- and concentration-dependent binding pattern of UK-101 in PC-3 cells.

Epoxyketones are known to be exceptionally specific for the Ntn-hydrolase class of enzymes. [193] Thus, additional targets of these small molecules are likely to be other proteasome subunits. Based on the results detailed above, we hypothesized that UK-101 binds proteasome subunits other than β1i. To test this hypothesis, we utilized a biotinylated probe of the proteasome, biotin-epoxomicin (Figure 4.9.A) in a competition assay. This is a well established method of detecting interactions with proteasome subunits by detecting the loss of binding between the subunit and the biotinylated probe after pretreatment with the compound of interest.

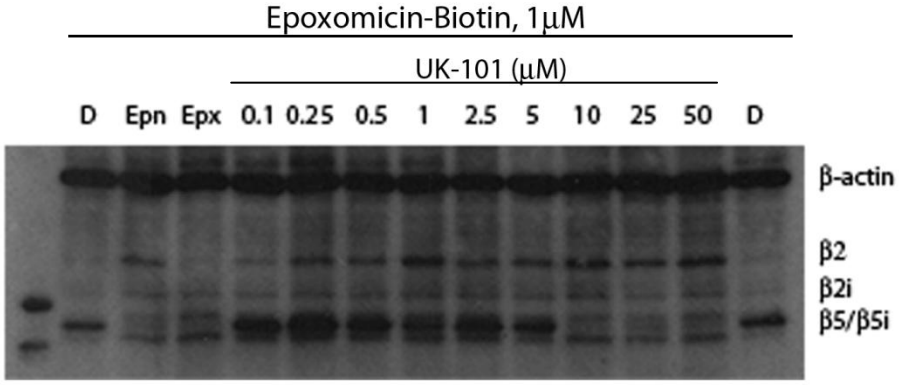
Using the same two hour treatment as in Figure 4.3, we examined the binding specificity of UK-101 over a large range of concentrations. Eponemycin and epoxomicin were used as positive controls and vehicle was used as a negative control. As seen in Figure 4.9.B there is no change in the binding pattern of epoxomicin-biotin at doses ranging from 0.1-5μM. However, at 10μM the strong band representing β5 and β5i diminishes significantly, suggesting that doses at and above this concentration are not specific for β1i. However, no additional changes in the binding pattern are seen between ten and fifty micromolar. If the cells were treated with compound for 24 hours, then exposed to biotinylated compound for an additional hour, a different picture emerges. As showed in Figure 4.9.C, UK-101 demonstrates a significant degree

A



Epoxomicin-Biotin

B



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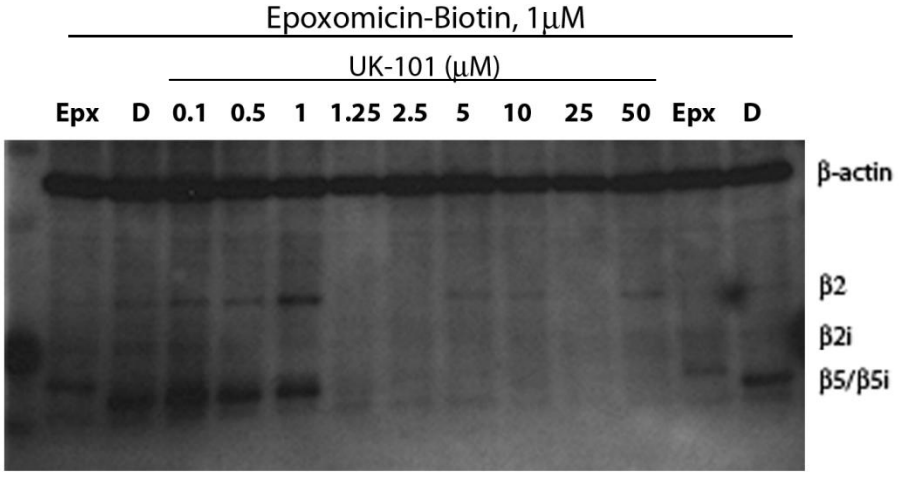


Figure 4.9: Competition assay to determine the proteasome binding specificity of UK-101. A. The structure of the probe, epoxomicin-biotin. **B.** The two hour competition experiment shows loss of specificity at high doses. Cells were pretreated with UK-101, DMSO (D), 1μM epoxomicin (Epx), or 1μM eponemycin (Epn). At the end of this treatment time, epoxomicin-biotin was added directly to the media and allowed to incubate for an additional hour. While UK-101 appears to have no effect on the β2 and β2i binding of epoxomicin binding, there is a reduction in the band intensity for β5/β5i at higher concentrations. **C.** Cells were treated as in **B** except that the first treatment was for 24 hours and the concentration of epoxomicin was reduced to 0.1μM. UK-101 is much less specific after 24 hours of treatment. At doses greater than one micromolar almost complete competition with the epoxomicin-biotin was observed, suggesting a significant loss in specificity over time in this continuous treatment paradigm.

of binding to other proteasome subunits at concentrations as low as 1.25μM. This is near the IC_{50} value for UK-101 and suggests that its growth inhibitory effect at 48 hours is likely due to the inhibition of multiple proteasome subunits.

These data, combined with the other studies detailed above, disprove the hypothesis that UK-101 is specific for β1i in the PC-3 prostate cancer cell line. Other groups, using β5 and β5i selective inhibitors, have recently observed a lack of cytotoxicity at subunit selective concentrations [92, 236, 238]. This evidence also suggests that the apoptotic effect of UK-101 may be due to the inhibition of multiple proteasome subunits. Thus, we attempted to address the question of whether β1i alone is a valid chemotherapeutic target by further studies in the PC-3 cell line.

F. Aim 2: Investigate the importance of β1i expression and function for proliferation in the PC-3 prostate cancer cell line

Since our small molecule probe of the β1i subunit is not truly subunit specific, we wanted to use alternative approaches to examine the importance of the β1i subunit and its catalytic function for the proliferation of the PC-3 prostate cancer cell line. Thus, we began by examining the effects of our siRNA model on PC-3 cell viability. Moreover, we produced a number of clonal cell lines expressing wild type or catalytically inactive β1i to examine its importance for cellular growth. Some of these clones contain the β1i gene under control of the tetracycline repressor, allowing us to "turn on" the gene upon addition of tetracycline or doxycycline to the media, providing temporal control.

With further medicinal chemistry efforts it may well be possible to produce a β1i selective, if not specific, epoxyketone proteasome inhibitor. However, examining the biological significance of β1i for the survival of PC-3 cells would provide additional information regarding the utility of a β1i selective inhibitor and suggest possible screening strategies for use during the validation of novel small molecule inhibitors of β1i.

G. Aim 2.1: Using a siRNA-mediated knock-down of β1i protein levels, determine the effect of loss of β1i on the viability of PC-3 cells.

As seen in Figure 4.6, siRNA treatment caused a significant reduction in β1i protein levels which was maintained over time. During this series of experiments, a difference in the confluence of

the cells was seen, as shown in Figure 4.10. This somewhat surprising result was observed repeatedly in a number of independent experiments. There were no apparent differences in cellular morphology, nor any differences in the number of detached cells between the groups. Thus, we hypothesized that the expression of catalytically active β1i promotes the proliferation of PC-3 cells.

To test this hypothesis we first did basic cell counting experiments to determine if this apparent difference was both real and significant. The first experiment confirmed that siRNA-transfected cells proliferated more slowly than mock-transfected cells, as expected, and the β1i siRNA treated cells proliferated more slowly than scrambled control treated cells (Figure 4.11.A). An independent experiment was performed to assess the significance of the difference in cell number between the control and knockdown cells, using two-way ANOVA. As shown in Figure 4.11.B, this difference was significant at the later time points of the experiment, with very high significance at 168 hours post-transfection.

While this exciting result suggested that β1i may play a role in the normal proliferation of PC-3 cells, this may also be due to an off-target effect of the siRNA. The siRNA used in the previous experiments was comprised of a pool of four independent oligos which all target different portions of the β1i coding sequence. This is generally accepted as the methodology which produces the best reduction in protein expression while minimizing off-target effects. [251] However, if one of these four oligos also had another target within the cell, it could be responsible for the proliferation change. Thus, we obtained the four individual oligos and tested them as single agents in our cells. As shown in Figure 4.12.A, all four oligos were able to knock down protein expression between 96 and 168 hours post-transfection, as was seen with the pool. When cell counting was performed with these individual oligos, however, none of the changes in cell number, when compared to the control siRNA, were shown to be statistically significant using two-way ANOVA (Figure 4.12.B). This suggests that the pooled knockdown cells, while indistinguishable from the individual oligos via western blotting, may produce a more complete reduction in protein expression, leading to the observed reduction in proliferation. We thus began further testing of this proliferation hypothesis.

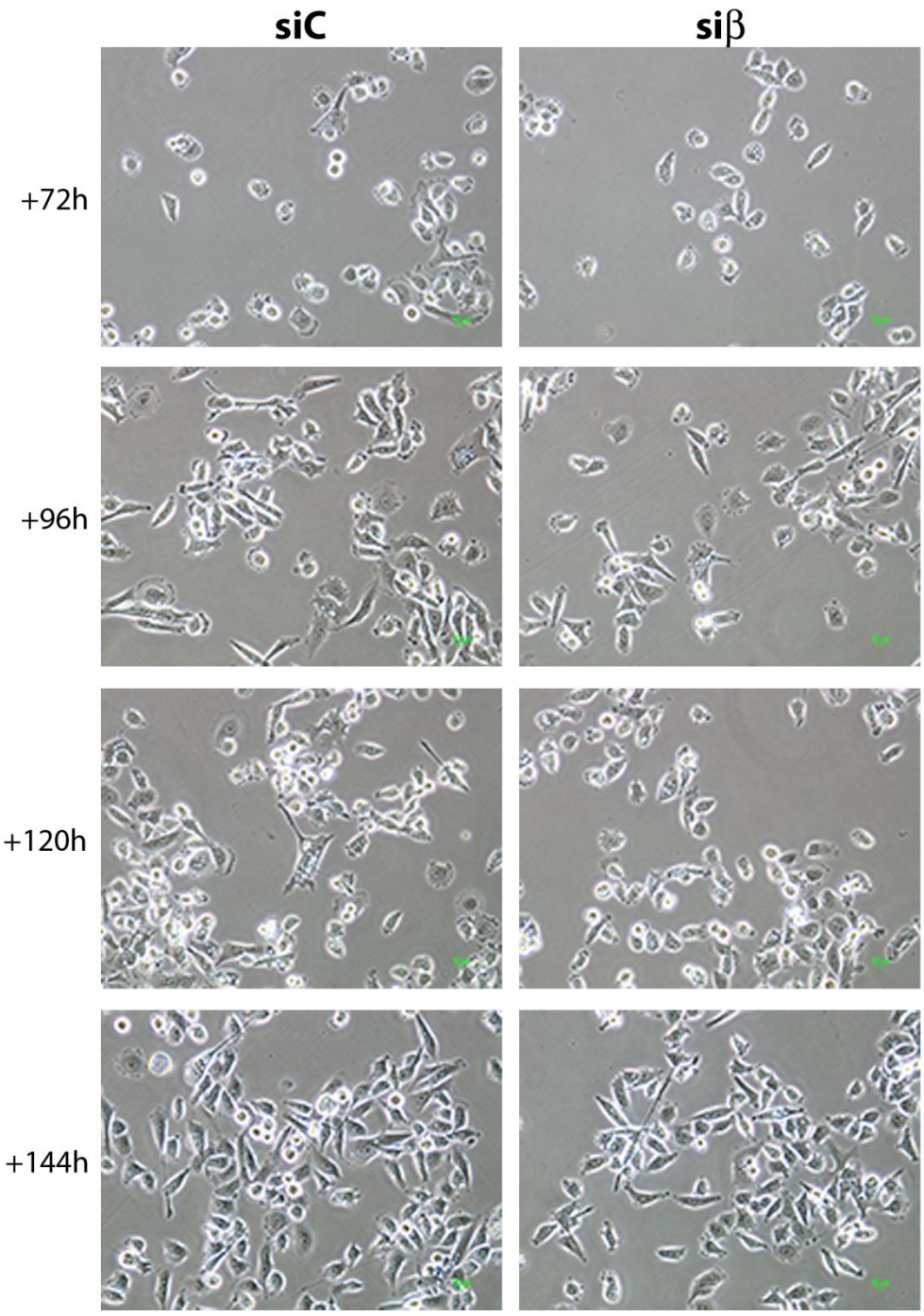


Figure 4.10: siRNA-mediated knockdown of β1i appears to affect the growth of PC-3 cells. Phase microscopy images of PC-3 cells taken at the times indicated post-transfection. There appear to be fewer cells in the scrambled control siRNA-treated cells were compared to β1i siRNA-treated cells. Otherwise, there seem to be no changes in cellular morphology between the groups.

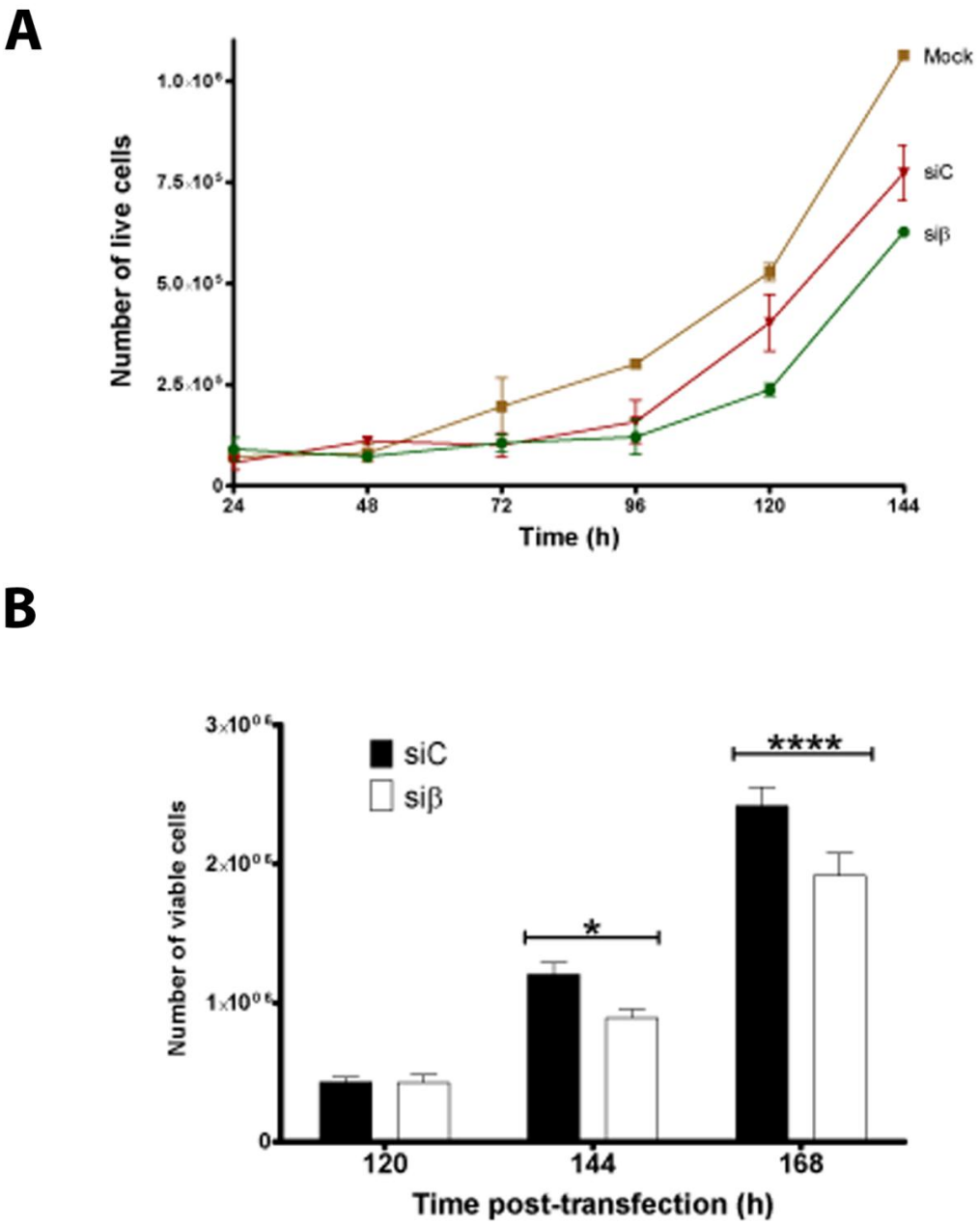


Figure 4.11: siRNA-mediated knockdown of β1i affects the proliferation of PC-3 cells. Cell counting of siRNA treated cells. **A.** Initial cell counting data showed a trend of decreasing cell number when scrambled control siRNA-treated cells were compared to β1i siRNA-treated cells. Both appear to have slower growth than mock-transfected cells. **B.** Cell counting data from a separate experiment showed that the difference between the scrambled control siRNA-treated cells and β1i siRNA-treated cells was significant (two-way ANOVA; * indicates $p<0.05$; **** indicates $p<0.0001$).

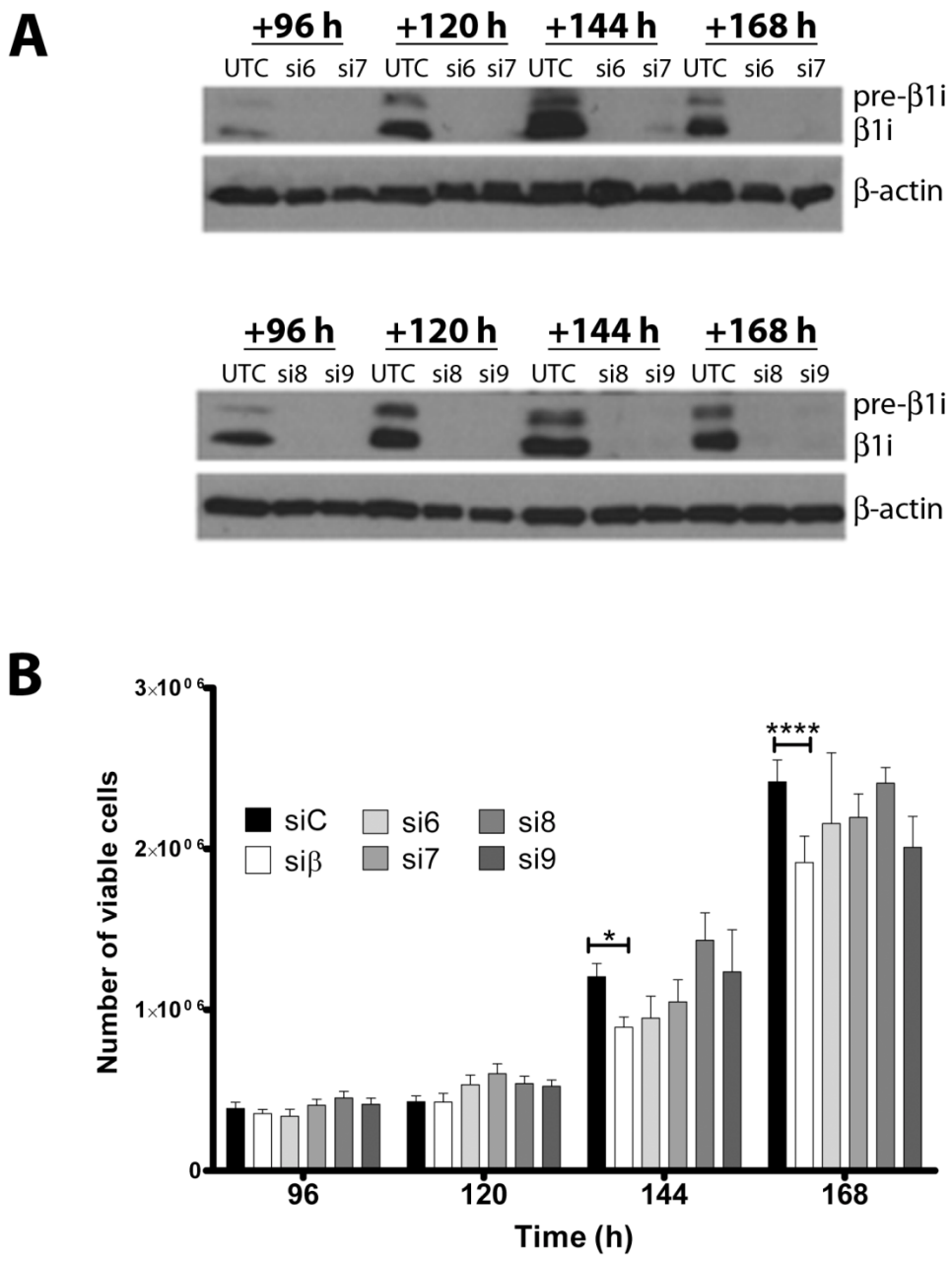


Figure 4.12: Individual siRNA oligos knockdown β1i but do not affect the proliferation of PC-3 cells. The individual oligos which made up the siRNA pool were purchased individually and treated to cells. **A.** Western blotting showed that the individual oligos were capable of significantly decreasing β1i expression over time, similar to the pooled siRNA. **B.** The individual oligos did not affect the growth of PC-3 cells in a significant way, as determined by two-way ANOVA, although the pooled knockdown from 4.11.B did (two-way ANOVA; * indicates $p<0.05$; **** indicates $p<0.0001$).

H: Aim 2.2: Using a plasmid-mediated overexpression of β1i protein levels, determine the effect of β1i and catalytically inactive β1i on the viability of PC-3 cells.

Natural inducers, such as IFN-γ and TNF-α, upregulate the expression of many other proteins in addition to β1i. To isolate the effect of upregulating β1i only, we used a plasmid containing pre-β1i, which should be correctly incorporated and activated by normal cellular processes. Transfection of DNA containing the entire protein, including the propeptide, is preferred over the mature protein so as to prevent random catalysis reactions and N-terminal acetylation, which inactivates the enzymatic function of the protein [63].

With this in mind, we utilized a dominant negative construct to replace the normal β1i in immunoproteasomes. This approach is an effective way to mimic the effects of enzymatic inhibition by a small molecule. Plasmids which express inactive mutant versions of the proteasome subunit β1i have been described in the literature [15, 18, 20, 23]. We created wild type and mutant forms of the β1i gene in two distinct constructs: first in a vector containing a myc-FLAG tag (Figure 4.13.A) and then in a vector containing the tetracycline operator sequence (Figure 4.13.B). The myc-FLAG system allows for the rapid detection and isolation of the gene of interest, although the tag could interfere with the protein's function. The tetracycline system allows for the induction or repression of gene expression based on the presence of tetracycline (or doxycycline) in the media. This is achieved by transfection with the plasmid (pcDNA6/TR) containing the tet repressor which, in this case, binds to the tetracycline operon in the absence of tetracycline to repress gene transcription (Figure 4.13.B). The mutant was created via site-directed mutagenesis and confirmed via sequencing (Figure 4.13.C), with the resulting mutation at amino acid 21 of the protein, which is amino acid one in the mature protein, as shown in Figure 4.13.D.

To first ensure the incorporation and activation of β1i, we utilized the myc-FLAG-tagged β1i plasmids. After transfection and selection with G418 (an aminoglycoside antibiotic similar in structure to gentamicin) for two weeks, we examined the expression of the myc tag using western blotting. As shown in Figure 4.14.A, the cells transfected with β1i-myc-FLAG express two proteins of the expected molecular weight corresponding to the inactive and active tagged form of β1i. The cells transfected with β1i-T21A-myc-FLAG, however, show a band of intermediate size in place of the active tagged form seen with the transfection of the wild type

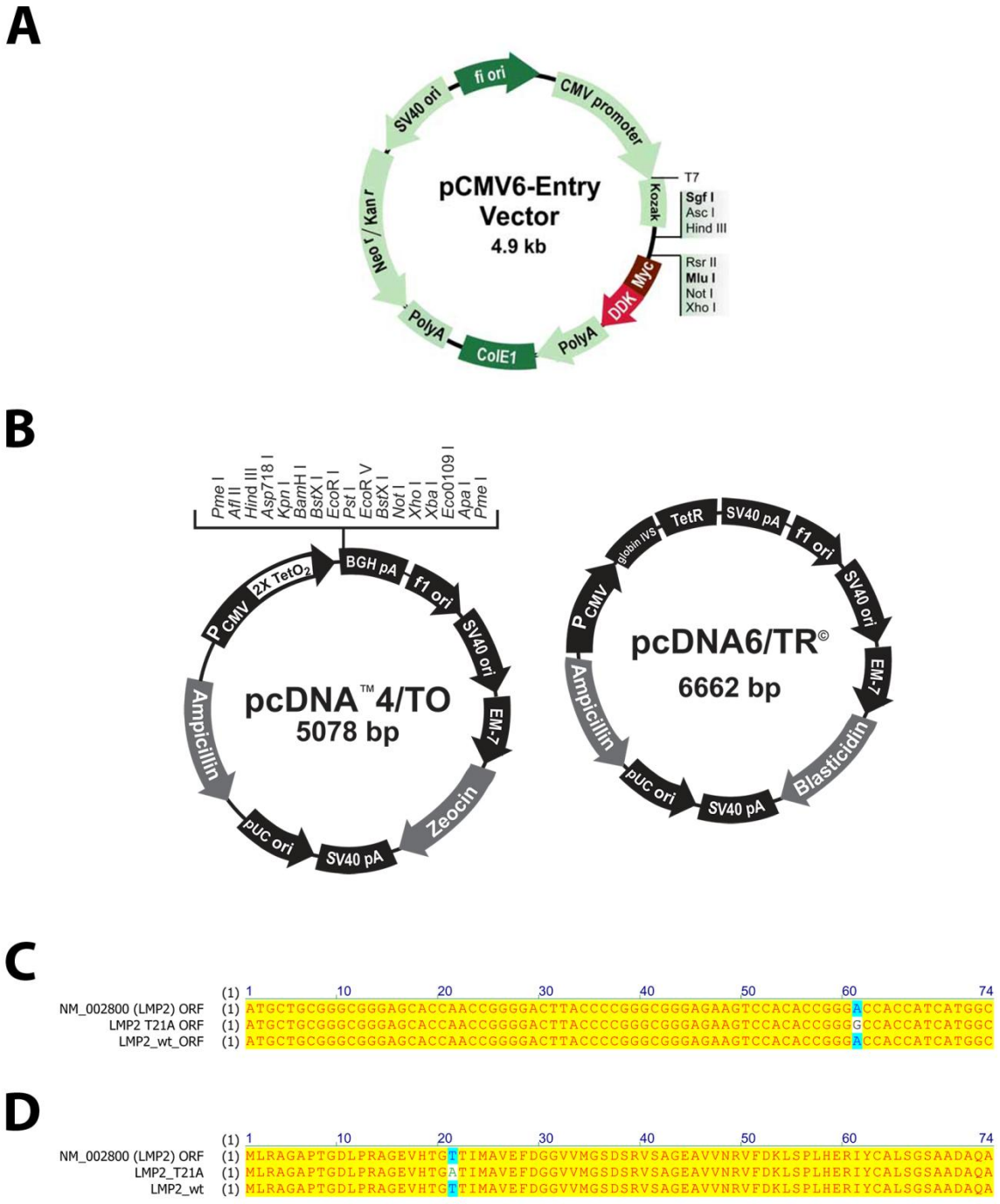
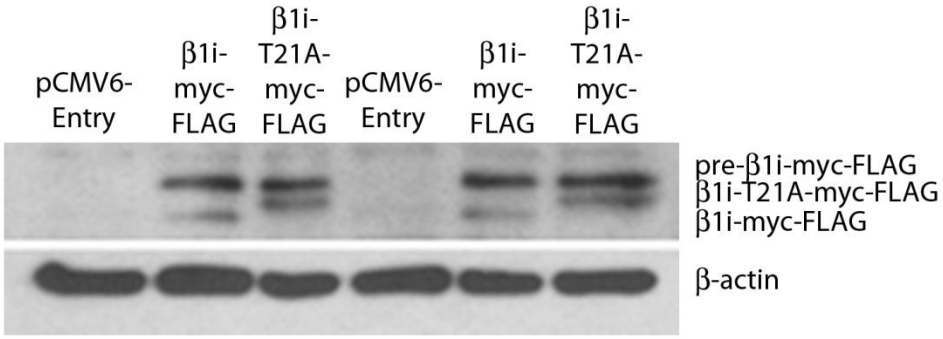


Figure 4.13: Vectors and sequences. A & B. The vectors used in the studies to follow. **C.** DNA sequencing results showing the correct mutation. **D.** Translation of the DNA sequence into protein shows the point mutation results in the correct amino acid change in β1i.

A


pCMV6-Entry
β1i-myc-FLAG
β1i-T21A-myc-FLAG
pCMV6-Entry
β1i-myc-FLAG
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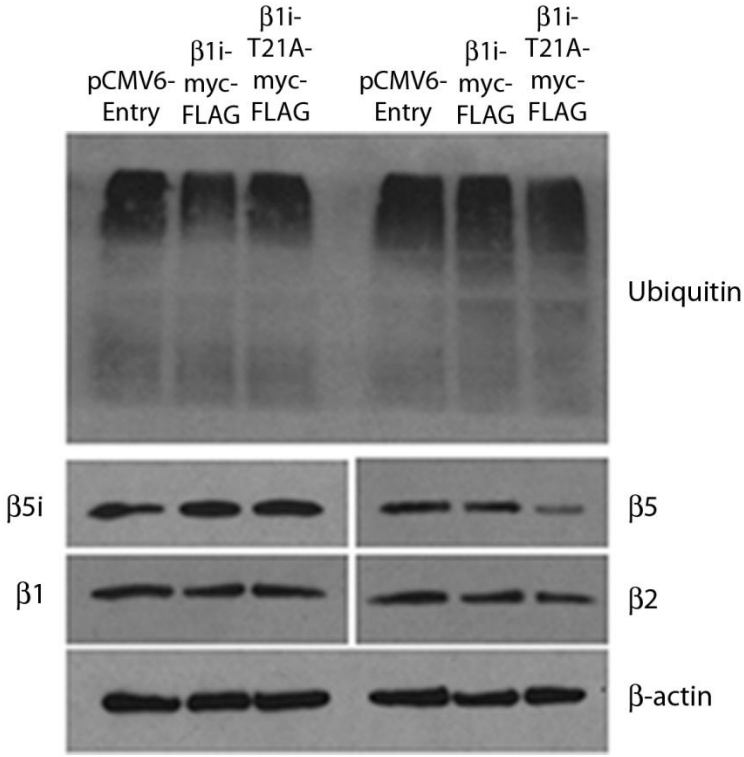
β1i-T21A-myc-FLAG
β1i-myc-FLAG
β-actin

B


pCMV6-Entry
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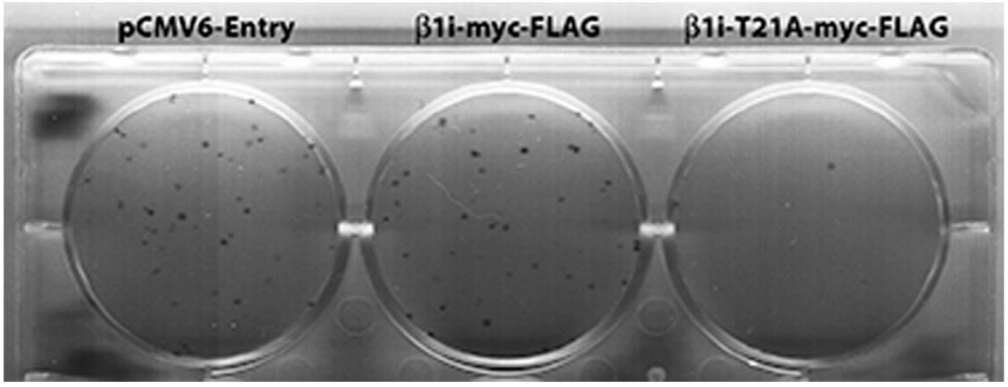
pCMV6-Entry
β1i-myc-FLAG
β1i-T21A-myc-FLAG

Figure 4.14: Expression the tagged catalytically inactive mutant of β1i appears to affect the growth of PC-3 cells. A. Western blot for the myc antibody showing expression of the unprocessed form of β1i in both the wild type and mutant transfected cells, but not those transfected with empty vector. The active form of the protein is present in both β1i-transfected cell types but at different molecular weights, due to changes in the propeptide cleavage. **B.** Detection of additional proteasome catalytic subunits and ubiquitin in these transfected cells**. C.** Colony formation assay showing a reduction in the ability of the β1i mutant transfected cells to grow into colonies when plated as single cells.

protein. This partially processed form should contain approximately nine or ten of the amino acids from the propeptide sequence, which are not cleaved due to the mutation of the threonine at amino acid 21 to alanine. [25, 81] Thus, our system is working properly, as both tagged forms of β1i are being incorporated into proteasomes, as shown by their processing into smaller proteins.

Further examination of these pooled cell populations showed no changes in overall levels of ubiquitin (Figure 4.14.B). However, there was a notable decrease in the expression of β5 in the cells transfected with the mutant β1i DNA (Figure 4.14.B). This may reflect increased proteasome turnover in these cells, as they attempt to compensation for the mutation in β1i which renders it catalytically inactive. There appears to be a slight increase of β5i expression in the β1i wild type and mutant transfected cells, as would be expected if the expression of β1i in the cells was increased (Figure 4.14.B).

Interestingly, after transfection and selection with antibiotics we found that there were many fewer surviving cells of the β1i-T21A-myc-FLAG variety than in the transfection with wild type β1i or empty plasmid DNA. We therefore took these pooled populations of selected cells and did a colony formation assay. As is shown in Figure 4.14.C, fewer colonies were seen in the mutant-transfected cell than the wild type or empty vector cells. These data suggest that constitutive expression of the catalytically inactive β1i mutant is detrimental to PC-3 cell proliferation.

To further test this hypothesis, we decided to use an inducible expression system, which would allow us to control the expression of the β1i gene using tetracycline or doxycycline. This would allow for the unbiased selection of cells containing the plasmid DNA rather than favoring the selection of cells which are resistant to the potential growth inhibitory or dependent on the growth stimulatory effects of the expressed gene. After creating stable cell lines expressing the tetracycline repressor protein using blasticidin and clonal expansion, we retransfected these cells with the β1i plasmids. We then selected these plasmids using zeocin, which is on its own promoter, and concurrently did a colony formation assay (Figure 4.15.A). This assay showed a great reduction in the number of colonies formed in the mutant cells which were induced when compared to their uninduced controls. Prior to clonal expansion, we did additional colony formation assays on the pooled cell populations by treating with or without doxycycline (compare bottom rows of Figure 4.15.B and 4.15.C). In this case, the number of colonies formed

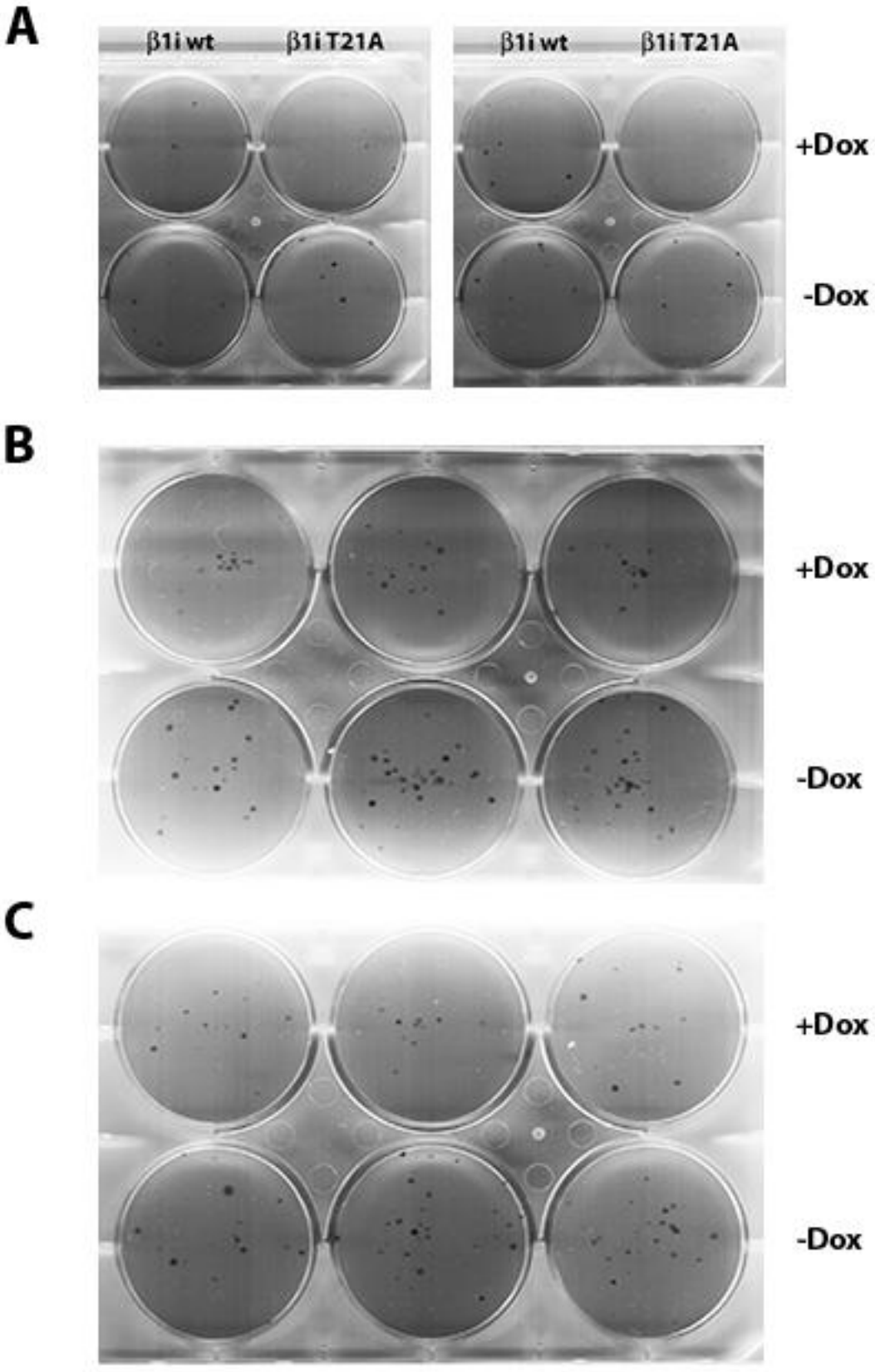
A
β1i wt
β1i T21A
β1i wt
β1i T21A
+Dox
-Dox
B
+Dox
-Dox
C
+Dox
-Dox

Figure 4.15: Expression the inducible catalytically inactive mutant of β1i appears to affect the growth of PC-3 cells. A. Colony formation assay during antibiotic selection of cells transfected with the tetracycline repressor protein and β1i wild type or mutant. Those cells selected with doxycycline in the media and the wild type protein have similar numbers of colonies while those with the mutant protein have few to no colonies. **B.** Pooled selected cells expressing wild type β1i were exposed to doxycycline or not until visible colonies were observed. The number and appearance of the colonies is less in the doxycycline-treated group. **C.** Pooled selected cells expressing mutant β1i were treated as in **B**. They show fewer colonies in the doxycycline treated group.

was more equal between the uninduced conditions. However, when the mutant β1i was expressed, there were fewer colonies seen and those observed appeared to be smaller than the uninduced control cells (Figure 4.15.C). The induction of wild type β1i also appeared to reduce the formation of colonies somewhat, although not as severely as the mutant (Figure 4.15.B). Thus, we decided to once again proceed with clonal expansion, so we could test the degree of induction with doxycycline against the proliferation rates of the cells.

Some of the clones generated were then tested for their ability to induce the expression of β1i. As shown in Figure 4.16.A, both clones were able to effectively induce expression of β1i after 24 hours at all doses of doxycycline tested. Additionally, this change in β1i expression had no effect on the levels of polyubiquitinated proteins in the cells. These two colonies were also tested to determine the effects of prolonged β1i induction on cellular proliferation. As shown in Figure 4.16.B, wild type β1i promoted the growth of the clone from Figure 4.16.A. Conversely, the mutant β1i did not promote the growth of the second clone from Figure 4.16.A; it actually appears to hamper the proliferation of this line somewhat (Figure 4.16.C). In the other clones tested, those which grew well showed similar trends as the clones depicted, although in many cases the differences were not significant due to a high level of variability between replicate wells.

Additionally, during this series of experiments we also transfected cells on a β1i-reduced background. However, we observed much more rapid growth in cells expressing β1i and transfected with these plasmids than in those that did not express much β1i. Thus, the data depicted here are cells grown on a parental background, which express a moderate level of β1i and are responsive to changes in β1i expression levels. Overall, these results suggest that β1i may be important for the proliferation of PC-3 cells. Further work is warranted to examine the generalizability of this response and further characterize the clones reported here.

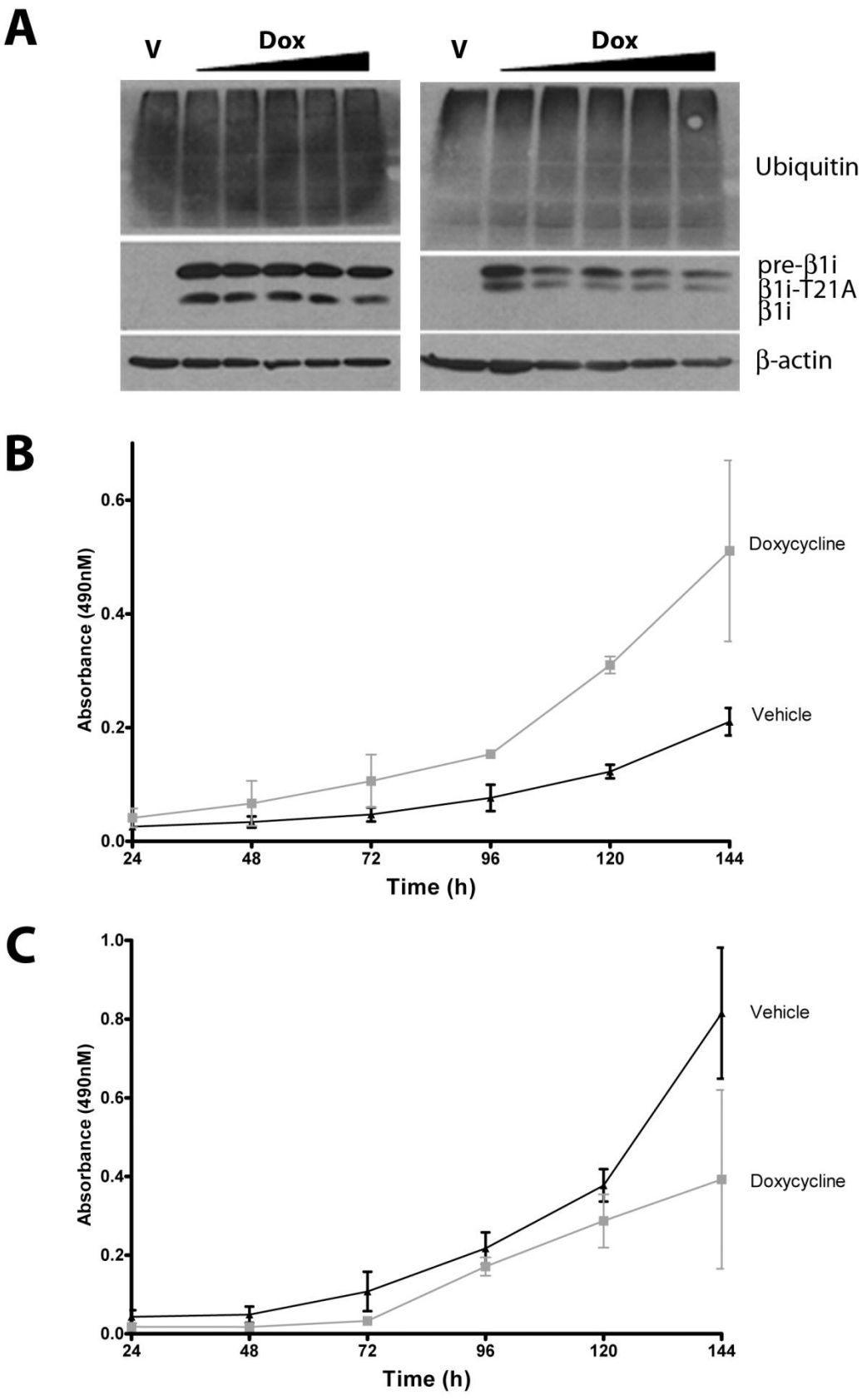
A
V
Dox
V
Dox
Ubiquitin
pre-β1i
β1i-T21A
β1i
β-actin
B
Absorbance (490nM)
0.0
0.2
0.4
0.6
24
48
72
96
120
144
Time (h)
Doxycycline
Vehicle
C
Absorbance (490nM)
0.0
0.2
0.4
0.6
0.8
1.0
24
48
72
96
120
144
Time (h)
Vehicle
Doxycycline

Figure 4.16: Expression of wild type β1i increases cellular proliferation while expression of the catalytically inactive mutant of β1i appears to decrease the growth of PC-3 cells. A. Western blotting from two clones of the same background, with the blot on the left having wild type β1i and the blot on the right having mutant β1i. The intermediate size of the partially processed mutant is shown on the right. Both clones give high induction of β1i upon addition of doxycycline with no change in the levels of polyubiquitinated proteins. **B.** Cell viability (MTS) assay with the wild type clone from **A**. Treatment with doxycycline (to induce expression of β1i) resulted in higher cell proliferation when compared to vehicle control. **C.** Cell viability (MTS) assay with the mutant clone from **A**. Treatment with doxycycline resulted in a slight decrease in cellular proliferation when compared to vehicle control.

Chapter 5: Conclusions & Discussion

A: Conclusions

The data described herein examine the utility of a β1i-targeting approach in prostate cancer. Using a small molecule inhibitor of the β1i subunit of the immunoproteasome, we began by examining its general effects on the PC-3 prostate cancer cell line. UK-101 caused apoptotic cell death in PC-3 cells after 48 hours (Figure 4.2) and likewise partially inhibited the proteasome after two hours (Figure 4.3). These data are complementary to and in line with previous work completed in our lab. [220, 223] They suggest that a β1i-targeting approach using UK-101 is a potential therapeutic strategy in prostate cancer. Since the development of novel chemotherapeutics is often hindered by off-target effects, we decided to utilize a target validation approach to confirm that UK-101 induces apoptosis through the covalent modification and inhibition of β1i. Thus, we began by examining the effects of modulating the expression of β1i on the sensitivity of PC-3 cells to UK-101.

First we examined natural inducers of the immunoproteasome, INF-γ (Figure 4.4) and TNF-α (Figure 4.5). Utilizing these cytokines is beneficial in that they are physiologically normal ways to cooperatively upregulate the expression of immunoproteasome catalytic subunits and ensure their maturation into fully functional proteasomes. However, these cytokines also have other cellular targets and effects, so information obtained in these experimental paradigms requires additional independent validation. Surprisingly, these two natural inducers gave opposite results, with the IFN-γ treated cells showing less sensitivity to UK-101 and the TNF-α treated cells showing more sensitivity to UK-101. This was our first indication that UK-101 may be acting on targets in addition to β1i.

To investigate this discrepancy, we utilized an opposing approach, reducing β1i expression levels. Then we examined the effect of treating these PC-3 cells with UK-101. Two complementary approaches were employed: a transient knockdown of β1i levels using siRNA (Figures 4.6 and 4.7) and a stable knockdown of β1i levels using shRNA (Figure 4.8). The major limitation of this approach was the dual functionality of the β1i protein, since it operates in both a proteolytic and scaffolding role for immunoproteasomes. Nevertheless, reducing the expression of β1i using either of these methods had no effect on the sensitivity of cells to UK-101. This was in direct conflict with our hypothesis and suggested that we should more directly

probe the interaction of this epoxyketone with other proteasome beta catalytic subunits, the most probable alternative targets for UK-101.

Thus, we examined the binding of UK-101 over two hours and 24 hours using the probe epoxomicin-biotin and found vastly different results (Figure 4.9). While the binding of UK-101 at two hours is relatively specific when examining physiologically relevant doses, after 24 hours this selectivity vanishes and loss of the β5/β5i band is observed. However, a molecular modeling study suggested that the tert-butyldimethylsilyl (TBDMS) group of UK-101 would have a strong steric clash with the S1 binding pocket of β5 [222]. This suggests that the molecule itself may change under prolonged exposure to aqueous conditions and cellular proteases.

With the specificity issue resolved, we moved forward to determine whether additional medicinal chemistry efforts to improve the stability and selectivity of UK-101 would provide a potential therapeutic molecule or simply a good molecular probe. We began by reexamining the siRNA model, in which we had observed an effect of β1i knockdown on cellular proliferation (Figure 4.10). Further experiments determined this effect to be statistically significant (Figure 4.11) and not due to a single oligo (Figure 4.12). However, it was impossible to determine using this approach whether the effect on cell proliferation was due to loss of the protein's catalytic function or loss of the protein's scaffolding function. Thus, we set out to examine the effect of introducing a catalytically inactive version of β1i into our cells.

The initial approach utilized was to transfect a tagged version of the wild type and mutant β1i to determine the extent to which β1i would be incorporated into proteasomes in the PC-3 cells. Incorporation of these tagged proteins would validate this approach, and lack of incorporation would suggest the necessity of removing the endogenous protein prior to replacement with the exogenous protein. Fortunately, the wild type and mutant proteins incorporate well (Figure 4.14) and we were pleasantly surprised to note the difficulty in culturing cells containing the mutant β1i. This suggested that it was the catalytic activity of β1i, rather than its structural function, which was driving the decreased cellular proliferation seen in Figures 4.10 and 4.11. It is difficult to do extensive experiments on cells which do not grow well and doing so is a selective process of its own. Therefore, we decided to place our β1i genes into an inducible expression vector, to complete the selection process without the confound of β1i gene expression enhancing or repressing cellular proliferation.

Once cell lines had been produced which stably expressed the tetracycline repressor, they were transfected with the inducible β1i expression plasmids. Induction of wild type gene expression during the selection process was a slight benefit while induction of mutant gene expression was detrimental to the cells (Figure 4.15). After selection, it was interesting to note that the pooled mutant cells displayed smaller colony sizes in general when compared to the wild type pooled cells, possibly due to leakiness of the repression system. Stable clones developed from these pooled cells were tested and found to rapidly and efficiently induce high levels of β1i expression when compared to endogenous β1i (Figure 4.16). This induction did not affect the overall levels of polyubiquitinated proteins. Clones also showed a similar trend in proliferation with and without induction as was seen in the pooled cell lines, with expression of wild type β1i being beneficial and expression of mutant β1i being neutral or detrimental (Figure 4.16).

These studies demonstrate that inhibition of β1i, whether chemically or genetically, has an impact on cellular proliferation. It is important to note this effect on proliferation is only seen after prolonged loss of β1i catalytic activity. Thus, small molecules which selective inhibit β1i may not have any effect after the short times normally examined, nor may they actually cause apoptotic cell death. Rather, it appears such molecules may function as growth inhibitory drugs for those cells which express β1i, assuming that near continuous inhibition is feasible over such long periods of time. A similar lack of apoptotic response was found when β5 or β5i selective inhibitors were tested in MM cell lines [92, 236]. This suggests that subunit specific proteasome inhibition may have unique characteristics, such as a lack of general cytotoxicity normally seen with less selective proteasome inhibitors. Future efforts to develop such subunit selective inhibitors should take these factors into account and not disregard small molecules which fail to quickly induce apoptosis.

The effects of catalytically inactive β1i are radically different than the characteristic effects of general proteasome inhibitors, or even activity-specific inhibitors. This suggests that, as noted above, subunit specific inhibitors will have unique functional effects, both *in vitro* and *in vivo*. Such a finding is exciting, as much remains to be understood in regards to the complex functional roles of individual catalytic beta subunits, but this implies that each catalytic subunit may have a distinct and meaningful role in normal and disease states. Thus, efforts to develop more selective and potent inhibitors of single proteasome catalytic subunits will assist in addressing such questions and should be encouraged.

B: Future Directions

Taken as a whole this work suggests that it is the catalytic activity of β1i which confers growth enhancing effects in PC-3 cells. It is important that further efforts examine the generalizability of this finding, especially between cancer cell lines or types. Additionally, examining the effects of β1i expression in *in vivo* cancer models would shed additional light on the clinical relevance of these findings and speak to β1i inhibition as a potential chemotherapeutic strategy. One way to do this would be by the development of fluorescent, luminescent, or near-infrared probes. Work is underway in the lab to do this using our lead compounds, although validation of these probes is still in the early stages.

The limitations of our small molecule inhibitor are not entirely surprising. Eponemycin, the parent compound of UK-101, binds preferentially to β1i, β5, and β5i, as UK-101 appears to do after 24 hours (Figure 4.9) [190, 191]. Thus, a simple *in vivo* deprotection of the P1' alcohol in UK-101 or cleavage of this bond by an esterase may be responsible for the loss of specificity of UK-101 over time. This suggests that derivation at the P1' position to remove the ester linkage while maintaining the overall structural bulk of the TBDMS group may improve the specificity of the compound significantly and should be explored in the future. However, additional validation work must be completed with any such new compound to ensure a lack of off-target effects.

Additionally, future medicinal chemistry efforts may benefit from exploring non-peptidic inhibitors, to alleviate additional proteolytic mechanisms of inhibitor inactivation. A collaboration with Dr. Chang-Guo Zhan has begun with the virtual screening of a library of approximately 250,000 small molecules to look for alternative chemical scaffolds which function as proteasome inhibitors. The ~200 lead compounds have already been selected and tested for their ability to selectively inhibit the immunoproteasome in the lab, utilizing purified proteasomes in an *in vitro* assay, and future work will involved cellular-based methods to confirm efficacy and examine permeability in cell line systems. Derivations of these novel scaffolds to produce selective proteasome inhibitors should improve *in vivo* stability by minimizing or removing the susceptibility of proteasome inhibitors to protease cleavage, an issue which currently limits the plasma half-life of carfilzomib to about 30 minutes [216].

Further studies to characterize the clones which express the inducible wild type and mutant β1i would allow for the examination of the downstream effects of loss of β1i function. We currently have no information regarding what occurs between proteasome inhibition and apoptosis, and this mutant induction system would allow one to address such questions without concerns regarding off-target effects of the treatment. This system can also quickly be utilized in other cell lines to examine the effects of inhibiting β1i, at least until a truly selective small molecule can be developed. Overall, these sorts of studies should also provide a more compelling rationale for the development of new molecular probes which selectively target β1i.

This inducible approach could also be used to examine mechanisms of resistance to β1i inhibition by comparing those clones which show growth inhibition upon induction of the mutant β1i with those that do not. Additionally, clones have been generated which are resistant to the effects of the tagged mutant, and these cells could likewise be compared to the vector and wild type clones to examine mechanisms of resistance to proteasome inhibition. This intrinsic resistance to partial inhibition of the proteasome is a problem clinically, and efforts to elucidate the mechanisms behind this resistance are underway in our lab and others.

As one potential mechanism of resistance to proteasome inhibitors is changes in proteasome subunit composition, the tagged β1i can also be used to quickly purify proteasomes from cells which are sensitive or resistant to partial proteasome inhibition. Since we have made no attempt thus far to determine the composition of the proteasomes in which our exogenous β1i is contained, such an approach will provide additional information regarding the cooperative assembly hypothesis, and possibly also provide purified intermediate proteasomes to test. This experimental approach could clarify and expand upon the western blotting results in Figure 4.14 and thus may be of interest for future studies.

The field of proteasome biology has come a long way from the days in which proteasomes were regarded as general garbage disposals, undruggable and uninteresting. As chemists develop more potent and selective inhibitors, biologists will continue to use them to make important discoveries which elucidate the functional complexity of the ubiquitin-proteasome pathway. Many unique roles of the proteasome and its catalytic subunits remain to be discovered, as we have barely begun to take into account the structural and functional intricacies of these complexes. Overall, the β1i-targeting approach remains promising, although there is much work left to be done to move it forward into preclinical development.

Chapter 6: References

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Vita

Marie Wehenkel

Born:

- December 20, 1983 in Sandusky, Ohio.

Education:

- Attended Terra State Community College.
- Attended Kent State University and obtained a Bachelor of Science (Magna Cum Laude) in Biological Chemistry in 2006.
- Attended the University of Kentucky.

Professional positions:

- Teaching Assistant, University of Kentucky.
- Research Assistant, University of Kentucky.

Scholastic and professional honors:

- Awarded a Trustee Scholarship from Kent State University for the years 2002-2006.
- Awarded a Founders Scholarship for Kent State University for the years 2002-2006.
- Awarded an Ohio Academic Scholarship from the Ohio Board of Regents for the years 2002-2006.
- Awarded a Graduate School Academic Year Fellowship from the University of Kentucky for the 2009 Spring semester.
- Awarded an American Association of Colleges of Pharmacy/Wal-Mart Scholarship for conference travel in the summer of 2009.
- Awarded a Kentucky Opportunity Fellowship from the University of Kentucky for the 2009-2010 academic year.
- Awarded an American Foundation for Pharmaceutical Education Fellowship for the 2009-2010 academic year, sponsored by the American Association of Pharmaceutical Scientists.
- Awarded a Presidential Fellowship by the University of Kentucky for the 2010-2011 academic year.
- Reawarded an American Foundation for Pharmaceutical Education Fellowship for the 2010-2011 academic year.
- Selected as a participant in the 2011 St. Jude National Graduate Student Symposium.

Professional Publications:

- Kimberly Cornish, Do-Min Lee, Ying Wu, Na-Ra Lee, **Marie Wehenkel**, Jason Lee, Beilei Lei, Chang-Guo Zhan and Kyung-Bo Kim (in press, 2011). “A bright approach to the immunoproteasome: Development of LMP2/β1i-specific imaging probes.” [Invited article, Chemical Proteomic Special Issue, Bioorganic Medicinal Chemistry.]
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- Yik Khuan (Abby) Ho, Paola Bargagna-Mohan, **Marie Wehenkel**, Royce Mohan, and Kyung Bo Kim (2007). "LMP2-Specific Inhibitors: Chemical Genetic Tools for Proteasome Biology." Chemistry & Biology **14**(4): 419-430.