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Investigating Mechanisms Determining Cancer Cell Sensitivity to Carfilzomib and Novel Strategies to Overcome Resistance

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INVESTIGATING MECHANISMS DETERMINING CANCER CELL SENSITIVITY TO CARFILZOMIB AND NOVEL STRATEGIES TO OVERCOME RESISTANCE

DISSERTATION

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

By
Lin Ao
Lexington, Kentucky

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ABSTRACT

INVESTIGATING MECHANISMS DETERMINING CANCER CELL SENSITIVITY TO CARFILZOMIB AND NOVEL STRATEGIES TO OVERCOME RESISTANCE

Proteasome inhibitors (PIs) are a class of FDA-approved anti-cancer agents which includes the first-generation PI bortezomib (BTZ) and second-generation carfilzomib (CFZ). Drug resistance is a major challenge in PI therapy with no solution currently available. While a few resistance mechanisms had been proposed for BTZ, little was known about CFZ resistance before the start of our studies. In this dissertation work, we investigated multiple mechanisms contributing to CFZ resistance—alterations in the drug transporter activity, metabolic stability, and proteasome activity profiles—and evaluated potential strategies to overcome CFZ resistance.

We observed marked upregulation of the drug efflux transporter P-glycoprotein (P-gp) in our H23 (lung cancer) and DLD-1 (colorectal cancer) cell line models of acquired resistance. P-gp inhibition by verapamil effectively restored CFZ sensitivity in resistant cells, indicating that P-gp contributes to CFZ resistance in our model. We designed a small library of CFZ analogs lacking the pharmacophore and screened them for their abilities to reverse CFZ resistance. Our results showed that dipeptide CFZ analogs were the most effective in restoring CFZ sensitivity. This study was among the first to demonstrate the involvement of P-gp upregulation in CFZ resistance and the feasibility of using CFZ peptide analogs to reverse P-gp-mediated CFZ resistance.

PI-resistant cancer cells often exhibit altered proteasome activity profiles compared to PI-sensitive cells. To further explore how these changes to the proteasome may influence cellular response to PIs, we developed a pancreatic cancer cell line model of acquired CFZ resistance. CFZ-resistant BxPC3 cells displayed a marked increase in the caspase-like (C-L) activity of the proteasome compared to parental controls. When challenged with CFZ, we also found that C-L activity was preserved in resistant cells whereas all activities were inhibited in parental cells. Using both chemical and genetic knockdown approaches, we found that co-inhibition of the C-L activity can sensitize resistant cells to CFZ. Similar effects

were also observed in CFZ-resistant RPMI-8226 multiple myeloma cells. These findings suggest that enhanced C-L activity may contribute to CFZ resistance and that combined inhibition of the C-L activity may serve as a potential strategy to restore CFZ sensitivity.

Since CFZ contains a tetrapeptide backbone and a highly reactive epoxyketone pharmacophore, its rapid metabolic inactivation *in vivo* may be a potential explanation for its lack of anti-cancer activity in solid cancers. Thus, we hypothesized that improving the metabolic stability of CFZ and its access to cancer cells may enhance its anti-cancer efficacy. Using micelle particles composed of biodegradable block copolymers poly-(ethylene glycol) (PEG) and poly-(caprolactone) (PCL), we demonstrated as a proof-of-concept that extended-release nanoformulations improved the metabolic stability and cytotoxic activity of CFZ in solid cancer cell lines. These findings supported the potential utility of polymer micelle formulations in enhancing the delivery of CFZ and improving anti-cancer efficacy CFZ against solid cancers.

Findings from this dissertation work enhance our understanding of factors contributing to CFZ resistance in cancer cells. Such information may be useful for the development of next-generation proteasome inhibitors and new strategies to combat CFZ resistance in the clinic.

KEYWORDS: Proteasome inhibitor, Carfilzomib, Resistance, Cancer

INVESTIGATING MECHANISMS DETERMINING CANCER CELL SENSITIVITY TO CARFILZOMIB AND NOVEL STRATEGIES TO OVERCOME RESISTANCE

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December 23, 2016

To my parents, who taught me to always work hard and never give up.

Thank you for your courage and inspiration.

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Chapter 1 RATIONALE FOR DISSERTATION WORK

(Each chapter in this dissertation contains its own introduction section, thus this chapter is intended to provide a brief rationale and overview for the entire dissertation.)

The ubiquitin-proteasome system (UPS) is a highly-regulated protein degradation system found in all eukaryotic life forms [1]. Its activity is crucial for maintaining protein homeostasis and regulating a myriad of cellular processes, including many of the ones involved in cancer pathogenesis [2]. Central to the UPS is the proteasome, a large multimeric protein complex consisting of regulatory particles and a 20S core which catalyzes the hydrolysis of substrate proteins in an ubiquitin-dependent manner [3]. Since its discovery over three decades ago, the proteasome has proven to be an important anti-cancer drug target. Initial validation of the proteasome as an effective anti-cancer target came with the blockbuster success of bortezomib (BTZ, Velcade®), a first-in-class proteasome inhibitor (PI) drug that revolutionized the multiple myeloma (MM) treatment paradigm [4]. Despite the clinical success, limitations associated with BTZ therapy, such as debilitating neurotoxicity and drug resistance, indicated that there was much room left for improvement [5]. To address some of the drawbacks associated with BTZ therapy, a second-generation PI carfilzomib (CFZ, Kyprolis®) was developed with a unique epoxyketone pharmacophore that bound to the proteasome selectively and irreversibly [6]. Preclinical and clinical evaluations demonstrated that CFZ had improved anti-cancer activity and better toxicity profile compared to BTZ [7, 8]. Based on these promising findings, CFZ received FDA approval in 2012 for the treatment of relapsed and refractory MM [9].

One of the major challenges to the continued clinical success of PI therapies is drug resistance [10]. Based on clinical assessments, both intrinsic and acquired resistance affected outcomes in the clinic: over 50% of MM patients receiving BTZ or CFZ as a single agent showed no disease improvement [11-14], and of the patients who initially responded, the majority eventually developed resistance [15]. Furthermore, cross-resistance to both BTZ and CFZ was also observed frequently in patients with relapsed and refractory MM and

effective treatment options were limited for patients who were resistant to both PI agents. In addition to resistance in hematological malignancies, patients with solid cancers also exhibited intrinsic resistance to BTZ and CFZ. This was in contrast to the compelling preclinical findings which demonstrated BTZ and CFZ having potent anti-cancer activities in models of solid cancer. The mechanisms responsible for this discrepancy between the preclinical and clinical findings were poorly understood, which limited further development of PIs as anti-cancer therapeutic for solid cancers [16, 17]. Thus, in order to enhance the anti-myeloma activities of BTZ and CFZ, as well as to expand their therapeutic potentials, it would be crucial to first gain an understanding of the mechanisms responsible for determining BTZ and CFZ resistance in cells.

At the beginning of this dissertation work, little was known about how PI resistance developed in cells and what factors were involved in determining cellular sensitivity to proteasome inhibition. Since BTZ had been in clinical use for nearly a decade, a few proposed mechanisms of BTZ resistance were available based on cell line models of intrinsic and acquired resistance. In contrast, nothing was known about CFZ resistance as it was not yet in clinical use. As it became evident that CFZ possessed superior anti-cancer activity and safety profile to BTZ, we felt that it was pertinent to gain a better understanding of the molecular mechanisms involved in determining cellular response to CFZ in order to exploit its full therapeutic potential. Thus, we set out to investigate the mechanisms responsible for determining CFZ sensitivity using cell line models of resistance.

This dissertation is consisted of three separate studies elucidating both proteasome-dependent and proteasome-independent factors involved in determining CFZ sensitivity. The studies described in chapters 3-5 are presented as separate chapters with unique leading hypotheses and background information, methods, results, and discussion sections. Chapter 2 provides a brief overview of the UPS and the development of PIs in the clinic, followed by a summary of the current understanding of BTZ and CFZ resistance, including mechanisms that were reported during and after the completion of the studies in this dissertation work. Discussion of the important questions that remain to be addressed in the field of PI resistance can also be found in the summary sections of chapter 2. Overall conclusions and implications of the studies presented in this dissertation can be found in

the final chapter (chapter 6), which also discusses future directions of our investigations as well as outlook of the field as a whole.

Each study presented in chapters 3-5 investigates a unique aspect of CFZ therapy and its potential contribution to CFZ resistance. Chapter 3 demonstrates upregulation of the efflux transporter P-glycoprotein (P-gp) as a major mechanism mediating acquired resistance to CFZ in DLD-1 colon and H23 lung cancer cells. In this study, we hypothesized that increased efflux transport of CFZ by P-gp conferred resistance to CFZ and that inhibition of P-gp activity could restore CFZ sensitivity in resistant cells. In support of our hypothesis, we found that overexpression of P-gp was associated with decreased CFZ sensitivity in colon and lung cancer cells and that inhibition of P-gp activity using small peptide analogs of CFZ reversed CFZ resistance. These findings were among the first to demonstrate a role for drug transporter activity in contributing to CFZ resistance.

During our investigation of P-gp-mediated CFZ resistance in Chapter 4, we noted changes in the expression and CFZ inhibitory profiles of several of the proteasome catalytic subunits in CFZ-resistant cells compared to CFZ-sensitive cells. These observations indicated that other mechanisms of resistance were likely contributing to CFZ resistance in addition to P-gp upregulation. This led us to investigate the role of altered proteasome activities in determining CFZ sensitivity using a P-gp-independent resistance model. In chapter 4, we hypothesized alterations in the baseline activities and inhibition profiles of the proteasome catalytic subunits contributed to CFZ resistance in BxPC3 pancreatic cancer and RPMI-8226 MM cells. We found that upregulated caspase-like (C-L) activity of the proteasome provided survival advantages in cells against CFZ-induced cytotoxicity, and that co-inhibition of C-L activity of the proteasome sensitized resistant cells to CFZ. Together, these findings support our hypothesis and indicate a previously unreported role for the C-L activity of the proteasome in determining cellular response to CFZ.

In Chapter 5, we carried out a collaboration study that addresses the poor metabolic stability of CFZ and its potential contribution to intrinsic resistance in solid cancer. We hypothesized that polymer micelle formulations of CFZ could improve its metabolic stability, thereby enhancing its anti-cancer efficacy. Our findings demonstrated as a proof-of-concept

six slow-release polymer micelle formulations of CFZ which achieved increased metabolic stability and remained active in H460 lung and RPMI-8226 MM cells. These findings support the potential utility of nanoparticle-based formulations as an alternative delivery method for CFZ.

In summary, the findings from this dissertation work contribute to our understanding of the factors involved in determining CFZ resistance, of which little was known at the beginning of our studies. Additionally, our work provides insight in the potential molecular targets and novel strategies that can be further developed to overcome resistance.

Chapter 2 INTRODUCTION

This chapter provides an overview of the development and clinical contributions of BTZ and CFZ, as well as a comprehensive review of the major findings reported to date contributing to the current understanding of BTZ and CFZ resistance. Additionally, we highlight here some of the proposed strategies to overcome PI resistance and discuss future directions of PI resistance research. Since the studies described in this dissertation began prior to the FDA approval of the third-generation PI agent ixazomib (IXA), and no resistance mechanisms have been reported for IXA so far, we mainly focus on the clinical activities and drug resistance of BTZ and CFZ therapies in this chapter. However, the BTZ and CFZ resistance mechanisms discussed here may also be applicable in understanding the molecular factors involved in determining cellular response to other PIs such as IXA.

2.1 The Ubiquitin-Proteasome System (UPS)

First discovered over three decades ago, the proteasome was initially thought to be the "garbage disposal" of the cell, with little function other than ridding the cell of defective proteins [18]. Since then, a tremendous expansion in knowledge about the UPS has drastically changed our perception of this protease complex [19]. With the help of new molecular tools and biochemical methods, the elucidation of the UPS opened our eyes up to the complex and intricate nature by which proteins are degraded inside the cell [20]. Along with that, we have also come to understand the fundamental importance of protein homeostasis and timely protein destruction to the livelihood of cells. The discovery and elucidation of the UPS, for which Drs. Hershko, Ciechanover, and Rose received the 2004 Nobel Prize in Chemistry [21], paved the way for the discoveries made in the following decades uncovering the regulation and function of biological processes such as apoptotic signaling, cell cycle progression, and the immune response. Moreover, their work contributed tremendously to our current understandings of diseases and ultimately led to the discovery of numerous life-saving therapeutics [22-24].

2.2.1 Controlled Protein Degradation

The UPS is responsible for the degradation and processing of more than 80% of all proteins inside the cell [25]. Protein degradation by the UPS is mediated by the covalent conjugation of a chain of simple molecules made up of 76 amino acid residues called ubiquitin [26]. Target proteins are usually tagged by long chains of ubiquitin moieties, called polyubiquitination, which are recognized by the proteasome and initiates their degradation [27]. The process of substrate-tagging with polyubiquitin moieties is carried out in a step-wise fashion by three classes of ubiquitin-interacting proteins. Ubiquitin ligase E1, also known as the ubiquitin activation enzyme, uses ATP hydrolysis to catalyze a thioester bond between itself and the C-terminal glycine residue of ubiquitin. Next, the ubiquitin molecule is transferred to the ubiquitin carrier protein E2 by a thioester bond transfer. Finally, the activated ubiquitin molecule is transferred to an E3 ubiquitin ligase protein, which both recognizes target proteins and conjugates ubiquitin molecules onto the substrates (Figure 2.1A). The polyubiquitinated protein is then recognized by the proteasome and degraded into small peptides [28, 29].

2.1.2 The Proteasome Complex

The quaternary architecture of the proteasome core is highly conserved among prokaryotic and eukaryotic cells, indicating that its function is indispensable [30, 31]. The proteasome consists of a barrel-shaped 20S core particle capped by a 19S regulatory particle on one or both ends of the core [31, 32]. Together, the 20S core with 19S particles associated at both ends make up the 26S proteasome (Figure 2.1B). The 19S regulatory particle is composed of at least nineteen subunits, nine of the which make up the lid and the other nine make up the base [33]. Subunits of the lid are responsible for the removal of the polyubiquitin chain from substrate proteins in an ATP-independent manner, whereas subunits are ATPases that recognize, unfold, and translocate substrate proteins to the proteasome core [34-37]. The 20S proteasome consists of four stacked heptameric rings: two outer α-rings and two inner β-rings. The α-rings form a gate to the β-rings, which ensures the sequestration of the catalytic subunits and prevents non-regulated degradation of cellular proteins [36]. Gate opening is regulated by the 19S regulatory particle, such that upon substrate binding, the 19S base subunits interact with the α-ring to trigger conformational changes in the α-ring and activate gate-opening mechanisms [38]. Other gate-opening mechanisms also include

the docking of other proteasome regulators onto the interface between the 19S and the α-ring. Following gate opening, the unfolded and deubiquitinated substrate protein can be translocated through the catalytically active β-rings for proteolysis [39].

The β-rings each contain three catalytically active subunits, β1, β2, and β5, that carry out the proteolytic functions of the proteasome complex [3]. Each of these β-subunits contains a catalytically active threonine residue at the N-terminus, making the proteasome a unique N-terminal nucleophilic peptidase capable of both catalytic attack and autocatalysis [40, 41]. During biosynthesis, each catalytic subunit is initially made with propeptides that protect the N-terminal threonine residue from acetylation and prevent premature proteolysis [42]. Upon complete assembly of the proteasome core, the propeptides are removed via autocatalysis as a final step to expose the active threonine residues of β1, β2, and β5 [33, 43]. The three catalytic β-subunits have different substrate preferences and carry out distinct proteolytic activities inside the cell. The β1 subunit, encoded by the *PSMB6* gene in humans, is referred to as having caspase-like (C-L) proteolytic activity due to its preference for peptide substrates containing acidic residues [44]. Conversely, the β2 subunit encoded by the *PSMB7* gene prefers substrates containing basic residues, and is consequently dubbed as having trypsin-like (T-L) catalytic activity [45]. The β5 subunit, encoded by the *PSMB5* gene, is equipped with chymotrypsin-like (CT-L) activity based on its preference for peptide cleavage at hydrophobic residues [46]. Together, these subunits cleave proteins into small peptides of unique and diverse sequences, which are especially important for antigen presentation and immune recognition [46]. In order to better understand the function of each catalytic subunit, chemical tools were developed to target each proteolytic activity which played an instrumental part in advancing our knowledge about proteasome biology and its role in disease pathogenesis [47].

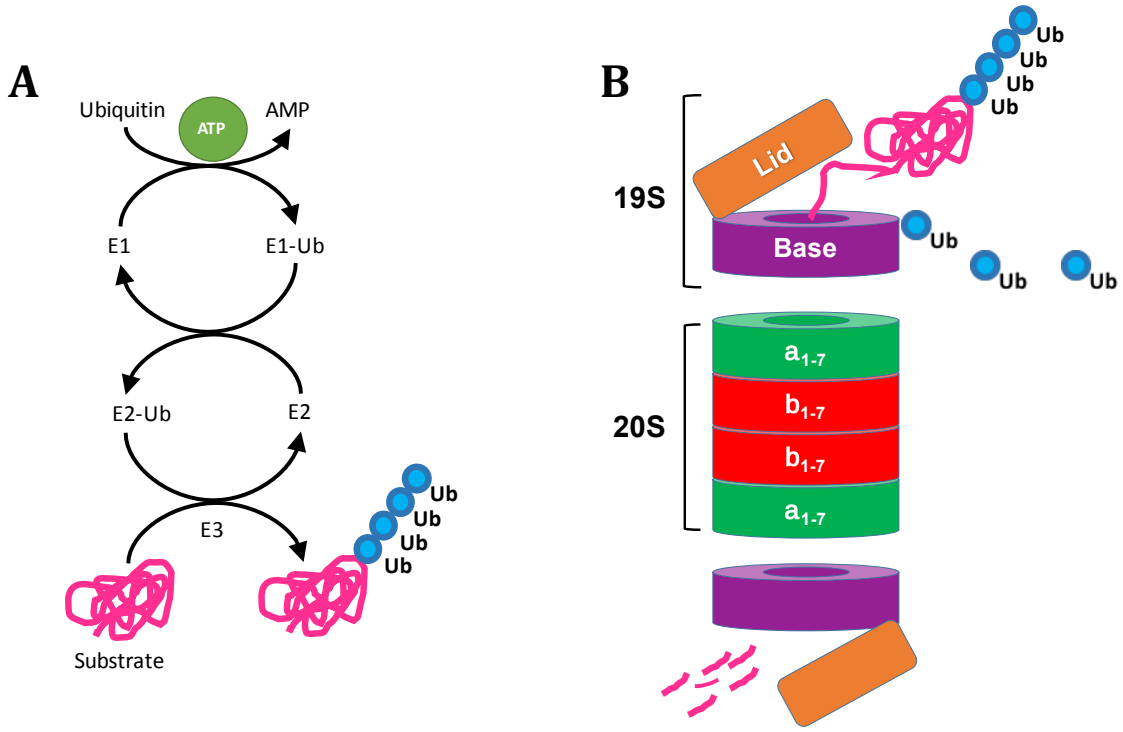

Figure 2.1 Proteolysis by the ubiquitin-proteasome system

(A) Ubiquitin is bound to an ubiquitin-activating enzyme E1, which transfers the ubiquitin molecule to a ubiquitin-conjugating enzyme E2. Finally, substrate protein is covalently attached to ubiquitin with the help of an ubiquitin ligase E3. (B) Structural representation of the 26S proteasome, consisted of a 20S catalytic core particle associated with two 19S regulatory particles. Substrate proteins are deubiquitinated by the lid subunits and unfolded and translocated into the 20S core by subunits of the base. Substrate proteins are degraded into small peptides by catalytically active β-subunits and released out of the proteasome.

2.1.3 Proteasome Subtypes

Three subtypes of proteasomes with unique structural and functional differences have been identified. These homologous subtypes of the proteasome differ mainly in their incorporation of a distinct set of catalytically active β-subunits in the proteasome core. As described above, the most common proteasome subtype containing β1, β2, and β5 is also known as the constitutive proteasome (CP), named for its ubiquitous expression in the cytoplasm and nucleus of all cell types [48]. CP activity is crucial in regulating a myriad of biological processes including cell proliferation, differentiation, and apoptosis. Its key roles in antigen presentation and immune function have also been well-described [49, 50].

Another major subtype of the proteasome, discovered decades after CP, is the immunoproteasome (IP). This proteasome subtype differs from CP by harboring a distinct set of structurally homologous catalytic subunits, β1i, β2i, and β5i, in place of β1, β2, and β5 [51, 52]. IP is named after its common expression in immune-derived tissues as well as the genes encoding β1i and β5i being in close proximity to the major histocompatibility complex II gene cluster [53]. Normally expressed abundantly in immune-derived tissues, IP expression is strongly induced during viral infection or other inflammatory responses by cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [50, 54, 55] (Figure 2.2). The exact functions of IP in cells is currently not understood. The presence of IP was initially thought to contribute to a wider diversity of antigenic peptides during viral infection and inflammatory conditions. However, as our understandings of IP functions evolved, non-immune related functions have also been discovered [56]. Both proteasome subtypes have been implicated in disease development. Cancer pathogenesis is the most well-studied due to the active development of proteasome-targeting small molecule inhibitors as anti-cancer agents. Other important disease implications of CP and IP function include viral and bacterial infections, autoimmune diseases, and aging [57-60].

A third proteasome subtype was found exclusively in the thymus and was thus named the thymoproteasome. This subtype is a derivative of the immunoproteasome and expresses a unique β5t catalytic subunit in place of β5i [61]. Similar to the immunoproteasome, the thymoproteasome contributes to mediating adaptive immune responses by introducing

distinct peptides to the antigen presentation repertoire. Its exact biological functions are not completely known; a number of recent studies have implicated thymoproteasome in cancer models [62]. However, as functions of the thymoproteasome and implications of the proteasome in non-cancer diseases are out of the scope of this dissertation, the remaining sections will only include the roles of CP and IP in cancer therapy and in PI resistance.

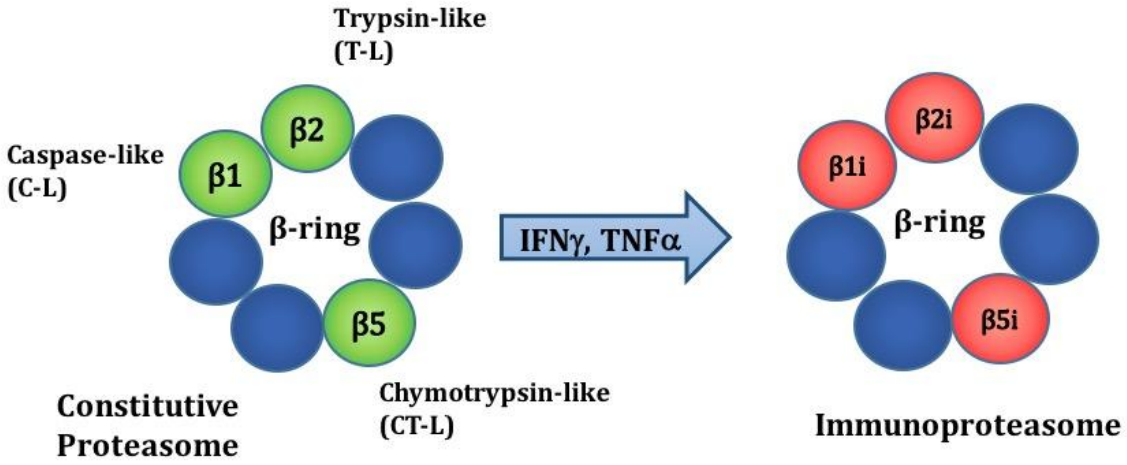

Figure 2.2 Catalytic subunit compositions of the constitutive proteasome and the immunoproteasome

Left: The constitutive proteasome (CP) contains catalytic subunits β1, β2, and β5 (green circles), which are recognized as having caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like activities (CT-L), respectively. Center: Immunoproteasome expression is inducible by cytokine stimulation, such as that by IFNγ and TNFα. Right: The immunoproteasome core particle differs from that of constitutive proteasome by the incorporation of homologous catalytic subunits β1i, β2i, and β5i (red circles) which possess slightly varied substrate specificities.

2.2 Bortezomib: First in Class

2.2.1 Validation of the Proteasome as an Anti-Neoplastic Target

As the role of the UPS became better elucidated throughout the 1990's, it became evident that the proteolytic role of the proteasome was crucial in the pathogenesis of an array of diseases including inflammatory diseases and cancer. Development of inhibitors targeting the proteasome began immediately following the elucidation of the UPS and its involvement in intracellular protein degradation [47]. The currently existing PIs are divided into five major classes—peptide aldehydes, β-lactones, vinyl sulfones, boronic acids, and epoxyketones—each distinguished by the unique warheads used by the PIs to attack the proteasome (Figure 2.3). Although the peptide aldehyde, β-lactone, and vinyl sulfone classes of PIs were not developed further as therapeutic agents due to toxicities and off-target effects associated with their use pre-clinically [63], their uses as biological tools were key to tease out the functional roles of the proteasome in regulating cellular pathways and cancer pathogenesis [25]. Crucial findings from these earlier investigations that demonstrated the potential of proteasome inhibition as an anti-cancer treatment included selective anti-cancer activity toward transformed cells over normal cells [64], synergistic cytotoxicity when combined with other chemotherapeutic agents [65], and sensitization of drug-resistant cancer cells [2].

Lack of specificity was one of the major drawbacks that prevented the clinical development of early PIs [66]. PIs such as the peptide aldehyde, β-lactone, and vinyl sulfone classes interacted with serine and cysteine proteases in addition to the proteasome [67]. Additionally, inhibition by these PIs could not distinguish between the different catalytic activities of the proteasome, which made it difficult to dissect their mechanisms of action inside the cells [57]. As an effort to improve the selectivity of the earlier PIs, derivatives of peptide boronic acids, which were known for their inhibitory activities toward serine proteases, were synthesized [68]. These derivatives were found to have up to 100-fold improved potency in proteasome inhibition and drastically enhanced selectivity toward the CT-L activity of the proteasome [68]. Thirteen of these peptide boronic acids were assessed in the National Cancer Institute (NCI) panel of 60 cancer cell lines for their anti-cancer activities. Among those tested, the dipeptide boron ester PS-341 demonstrated the most

potent proteasome inhibition and cell-killing efficacy [69]. Based on these findings, PS-341 was further investigated as a potential anti-cancer therapeutic, and was later re-designated as bortezomib (BTZ).

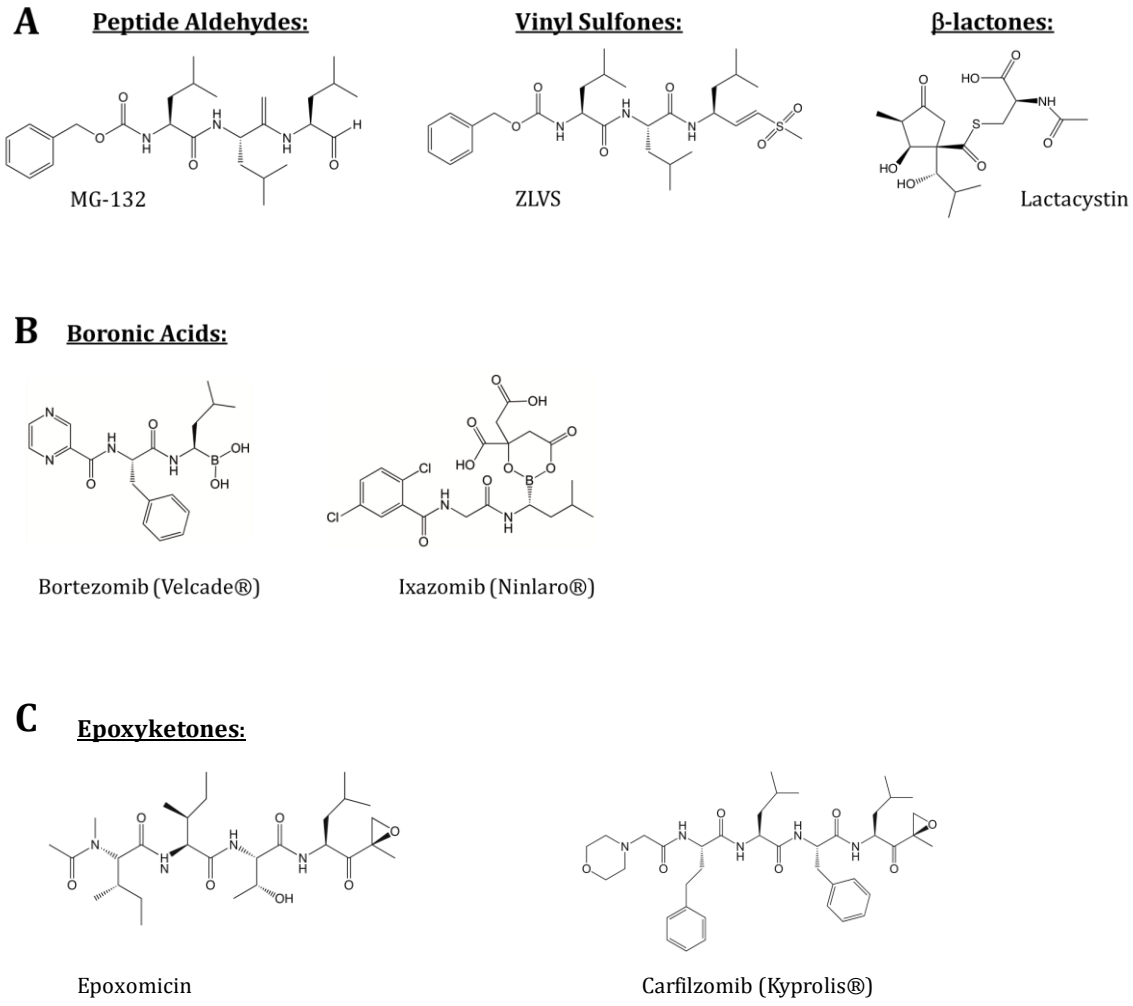

Figure 2.3 Proteasome inhibitors as research tools and clinical agents.
Examples of the five classes of proteasome inhibitors currently in development or clinical use. (A) Early proteasome inhibitors MG-132, ZLVS, and β-lactone belong to the peptide aldehyde, vinyl sulfone, and β-lactone classes of proteasome inhibitors, respectively. These agents were not pursued for clinical development but remain important tools for assessing proteasome biology (B) The boronic acid class includes two of the currently approved proteasome inhibitors bortezomib (Velcade®) and ixazomib (Ninlaro®). (C) The epoxyketone class includes the natural product epoxomicin and the second-generation proteasome inhibitor drug carfilzomib (Kyprolis®).

2.2.2 Preclinical Development

BTZ is a slowly-reversible inhibitor that preferentially binds to the β5/5i proteasome subunits, and the β1i subunit to a lesser extent [57, 70]. Currently, the exact mechanisms through which BTZ induces cell death are not completely known. Early preclinical findings indicated that accumulation of the cell cycle inhibitor protein p21 and blockade of G_1-S and G_2-M cell cycle transitions contribute to BTZ-induced apoptosis [69]. Stabilization of the tumor suppressor protein p53, which is a known substrate of proteasome degradation, has also been shown to have a role in mediating BTZ-induced cell death in some cases [71, 72]. However, whether p53 function is required for BTZ-induced cell death is controversial, as other studies have also found that BTZ can activate apoptosis in cells regardless of p53 status [69, 73]. Other mechanisms attributed to mediating BTZ-induced cell death also include inhibition of NFκB transcription [74], activation of the unfolded protein response [75], and upregulation of pro-apoptotic proteins [76].

Proteasome inhibition by BTZ yielded nanomolar cell-killing potencies against hematological and solid cancer cell lines [69], including multiple myeloma [77], mantle cell lymphoma [73], non-small cell lung cancer [78], and colorectal cancer [79]. BTZ treatment also induced growth arrest and apoptosis in primary MM cells as well as RPMI-8226 MM cells with resistance to doxorubicin and melphalan [80-82]. Consistent with these *in vitro* findings, BTZ also demonstrated potent anti-cancer activity *in vivo*. Intravenous administration of 0.3 mg/kg or 1.0 mg/kg BTZ in mice bearing xenografts of human PC3 prostate cancer cells resulted in dose-dependent reduction of tumor volumes and inhibition of proteasome activity in the tumor tissue [69]. Similar observations were also reported in mouse xenograft models of lung cancer [83], pancreatic cancer [84], and MM [85]. The comparable BTZ potencies observed among these models led researchers to believe that BTZ would be equally effective for treating both hematological and solid malignancies.

Proteasome inhibition resulting from BTZ was detected in white blood cells of rats, as well as a number of peripheral tissues, including liver, kidney, prostate, adrenals, with limited penetration into the central nervous system [69, 86]. BTZ metabolism by human liver microsomes indicated that oxidative deboronation was mainly carried out by liver P450

enzymes including CYP3A4, CYP2C19, CYP1A2, CYP2D6, and CYP2C9 [87]. Initial assessment of toxicity in mice bearing prostate xenografts found no major adverse effects associated with either 0.3 mg/kg or 1.0 mg/kg of BTZ administration [69]. Additional preclinical evaluations in rodents also found BTZ treatment to be generally well-tolerated [88]. However, gastrointestinal side effects were reported as main toxicities associated with twice-weekly intravenous administration of BTZ in primates [88]. Studies in primates also established a dose of 0.8 mg/m^2 as the maximum tolerated dose, which was taken into account for clinical assessments of BTZ [16]. Collectively, these findings demonstrated the potential of BTZ as an anti-cancer agent and provided the framework for its clinical development.

2.2.3 Clinical Development & FDA Approval

Phase I clinical studies of BTZ evaluated three dosing regimens of intravenous BTZ: once weekly for four weeks, twice weekly for two weeks, and twice weekly for four weeks. Maximum tolerated doses ranged from 1.04 mg/m^2 for the most intensive schedule (twice weekly for four weeks) to 1.56 mg/m^2 for the least intensive schedule (once weekly). BTZ treatment was well-tolerated by patients in general, with major toxicities reported including thrombocytopenia, fatigue, and peripheral neuropathy [16, 89]. Based on these findings, subsequent phase II clinical trials were designed to evaluate the safety and efficacy of BTZ in patients with relapsed and refractory MM [90, 91]. These clinical evaluations established a dosing schedule of intravenous 1.3 mg/m^2 BTZ administered twice weekly and demonstrated overall response rates of 35% and 38% for single-agent BTZ in patients with relapsed and refractory MM [16, 89-92].

In addition to single-agent evaluations, BTZ also demonstrated remarkable anti-myeloma activity when administered in combination with other agents including dexamethasone, thalidomide, and doxorubicin. Importantly, inclusion of BTZ in these combinational treatments significantly improved anti-myeloma efficacy in newly diagnosed and relapsed and refractory MM patients compared to treatments without BTZ [93-95] . Moreover, BTZ monotherapy also demonstrated significant activity in extending the response duration and overall survival of patients with mantle cell lymphoma [96]. Together, these findings led to the FDA approval of BTZ in 2003 for the treatment of relapsed and refractory multiple

myeloma [97]. Additional clinical evaluations following its initial approval prompted the expansion of its label to include previously untreated multiple myeloma and mantle cell lymphoma [98, 99].

2.2.4 Limitations

Despite the recent advances gained in anti-myeloma therapy with the introduction of BTZ, MM remains an incurable disease due to disease relapse and lack of efficacy in a subset of patients [100]. According to a study by Richardson et al., only 41% of previous untreated MM patients achieve partial response or better with single-agent BTZ treatment, indicating that intrinsic resistance to BTZ affects over half of BTZ-naïve patients [12]. In addition to intrinsic resistance, acquired resistance occurs inevitably in most patients, as the median duration of response in patients was found to be approximately 12 months [12, 14, 101, 102]. Effective treatments for PI-resistant MM patients are currently limited, thus development of novel therapies to combat resistance are desperately needed. One of the major challenges to the development of therapies to overcome resistance is the poor understanding of the mechanisms underlying the development of BTZ resistance. Several mechanisms have been proposed over the last decade, some of which have been cross-validated in multiple models of resistance. However, most of these findings were identified in cell line models and evidence supporting their clinical relevance is lacking in general. Investigations are ongoing to uncover novel mechanisms responsible for BTZ resistance as well as to examine the clinical importance of the currently known mechanisms. A summary of the major findings contributing to the current knowledge about BTZ resistance can be found in sections 2.6-2.8.

In addition to drug resistance, another major drawback associated with BTZ therapy is the dose-limiting peripheral neuropathy, observed in up to 52% of MM patients receiving BTZ-based therapy [5, 103]. This neurotoxic effect of BTZ is believed to be associated with off-target interactions of the boronic acid pharmacophore with the serine protease HtrA2/Omi that is crucial for neuronal survival [104]. Since the severity of peripheral neuropathy in BTZ-receiving patients is directly correlated with BTZ dosage, the therapeutic window of BTZ is limited to a narrow range of 1.0mg/m^2 to a maximum tolerated dose of 1.5mg/m^2 [89]. Additionally, the toxicities associated with BTZ treatment also limit its administration

to once every three days, making modifications to its dosing schedule difficult. In regards to this, a recent phase 3 clinical found that subcutaneous administration of BTZ achieved similar anti-myeloma efficacy compared to intravenous BTZ but was associated with lower incidents of peripheral neuropathy; grade 3 or higher peripheral neuropathy was detected in 57% of patients in the subcutaneous group compared to 70% in the intravenous group [105, 106]. Based on these findings, subcutaneous administration was recently included into the FDA prescribing information of bortezomib [107].

2.2.5 Summary

Prior to the development of BTZ, there was skepticism regarding the feasibility of targeting the proteasome as a therapeutic option [108, 109]. It was assumed that shutting down the activity of a protein that is ubiquitously expressed throughout the body and is considered vital to cell survival would result in catastrophic outcomes [22]. As it turned out, this was not the case. The FDA approval of BTZ validated the proteasome as not only a feasible, but an extremely effective target for cancer pathogenesis. The introduction of this first-ever PI agent brought unprecedented therapeutic benefits in the outcomes of MM therapy, and paved the way for a whole new territory of therapeutic exploration within the UPS. The proteasome inhibitors were no longer viewed as mere tools for biological probing, and with BTZ came a newfound enthusiasm within the field to develop next-generation PI agents to improve upon the limitations of BTZ [110]. As a result, two more PI agents were added to the MM arsenal in the decade following BTZ's approval and several more are currently undergoing clinical assessment [111].

2.3 Carfilzomib: The Next Generation

2.3.1 Overview

The second-generation PI agent carfilzomib (CFZ, Kyprolis®) is currently approved for use as a monotherapy or in combination with immunomodulatory agents to treat patients with relapsed and refractory MM who have received one to three prior treatments [112]. Its fast-track approval in 2012 was supported by the potent anti-cancer activity and favorable toxicity profile CFZ demonstrated in preclinical and clinical studies. Since its initial approval, which restricted its use as a third-line therapy, CFZ has gained additional FDA approvals to fully expand its label in 2016 to include first-line indications for the treatment

of relapsed and refractory MM [113, 114].

These subsequent approvals came on the heels of recently published clinical findings supporting the use of CFZ as a superior alterative to BTZ both as a monotherapy and in combination with immunomodulatory agents. When compared head-to-head with the first generation PI, CFZ-based therapy achieved significantly higher progression-free survival and overall response in compared to BTZ-based therapy in relapsed MM patients [113, 115]. Importantly, CFZ therapy remained efficacious in a significant portion of BTZ-resistant MM patients, indicating that CFZ therapy can be used to overcome BTZ refractory disease [11]. For these reasons, CFZ has gained recent recognition as a superior potential alternative to BTZ in the MM standard of care [116]. Furthermore, the favorable efficacy and toxicity profiles of CFZ also makes it a more desirable candidate for other disease implications, including non-hematological malignancies and other inflammatory conditions [6, 117, 118]. Strategies to expand its clinical utility in these regards are currently under investigation.

Despite having advantages over BTZ in efficacy and toxicity, CFZ therapy has its own set of limitations. Among these, drug resistance represents a major roadblock in the lasting clinical success of CFZ therapy. Similar to BTZ therapy, both intrinsic and acquired drug resistance also prevent CFZ from reaching its full therapeutic potential. Furthermore, much less is currently known about the mechanisms underlying CFZ resistance compared to BTZ resistance. Thus, it is critical to understand the factors involved in determining CFZ response, and how these mechanisms may differ from those dictating BTZ sensitivity. Such information will not only allow us to develop strategies to predict and improve CFZ response in the clinic, but will also be useful for the further development of CFZ as a treatment for other cancers and diseases. Additionally, insights gained in elucidating mechanisms involved in BTZ and CFZ resistance can provide important clues for the design and development of next-generation PIs.

2.3.2 A Structurally Distinct Second-Generation PI

CFZ differs from BTZ in its tetrapeptide backbone structure and distinct epoxyketone pharmacophore, which allows CFZ to bind to the proteasome in a highly selective and non-

reversible manner [119]. Specific inhibition of the proteasome active site is mediated through a unique interaction between the epoxyketone moiety and the N-terminal threonine active residue of the proteasome catalytic site. Through a two-step nucleophilic attack reaction, CFZ binds to the proteasome irreversibly through the covalent formation of a seven-membered ring adduct [119]. Given that this mechanism is only made possible by the participation of a protease with an N-terminal active threonine, the epoxyketone warhead is considered to be much more proteasome-selective than other PI pharmacophores.

The structure and design of CFZ was derived from the natural product epoxomicin, which was isolated in the late 1990's and found to have proteasome inhibition and anti-inflammatory activities in cells [120, 121]. Although epoxomicin proved to be a useful tool in identifying the proteasome as its major target of inhibition, its low cell-killing potency and broad inhibition of proteasome catalytic activities prevented its further development as a therapeutic agent. Building upon the structure of epoxomicin, medicinal chemistry efforts produced several other epoxyketone-containing compounds in hopes of identifying a lead with high inhibitory potency and selectivity toward the proteasome [122]. Among these epoxomicin derivatives, the compound YU-101 demonstrated both higher proteasome selectivity and improved anti-tumor potency compared to both epoxomicin and BTZ [8]. Further modifications to the N-cap moiety of YU-101 resulted in improved solubility and yielded the compound PR-171, later re-designated as CFZ [8, 123].

2.3.3 Preclinical Development: Lessons Learned from BTZ

CFZ mainly inhibits the CT-L (β5/5i) activities of the proteasome, with better selectivity toward the β5 subunit and decreased interactions with the β1i subunit compared to BTZ [7, 124]. The unique epoxyketone pharmacophore of CFZ also decreases its off-target interactions with other non-proteasomal proteases, an important improvement from BTZ [104]. CFZ successfully induced polyubiquitinated protein accumulation and cell cycle arrest with nanomolar potency in cancer cell line models of hematological and solid malignancies. Additionally, brief exposure of CFZ was found to be more cytotoxic than BTZ in MM cells, likely due to the irreversible binding of CFZ to the proteasome [6]. Cell death resulting from CFZ-mediated proteasome inhibition has been associated with activation of

the c-Jun N-terminal kinase, mitochondrial depolarization, and activation of both intrinsic and extrinsic apoptotic pathways [125]. *In vivo* evaluations of CFZ efficacy in mouse xenograft models further verified its potent anti-cancer activity [126]. Importantly, CFZ remained active in MM cell lines and tumor cells from BTZ-refractory patients and cells resistant to conventional chemotherapeutics, suggesting that the mechanisms of CFZ resistance may be unique from those of BTZ and conventional chemoresistance [125]. With these promising preclinical findings, clinical evaluations of CFZ quickly proceeded to determine its activity and safety in patients with both hematological and solid malignancies.

2.3.4 FDA Approval & Clinical Success

CFZ was initially evaluated in two Phase I clinical trials for activity and safety in patients with refractory hematological malignancies that were treated with at least two prior therapies. Results from these studies established a consecutive-day, twice weekly dosing schedule of CFZ at doses up to 27 mg/m^2 administered by intravenous injection. CFZ was well-tolerated across different doses and an overall response of partial response or better was observed in 15 of 37 patients treated with CFZ in the two trials [127, 128]. These findings led to additional phase 2 clinical trials evaluating the activity of single-agent CFZ administered intravenously at 20 or 27 mg/m^2 in patients with relapsed and refractory MM who have failed two prior therapies, including BTZ and one immunomodulatory agent (e.g. thalidomide and lenalidomide) [11, 13]. Overall response rates observed were 13% for 20 mg/m^2 CFZ and 24% for 27 mg/m^2 CFZ, with an average duration of response of 7.4 months [11, 129]. Together, findings from these phase 2 studies provided the basis for the accelerated FDA approval of CFZ in 2012. This initial approval granted use of CFZ for the treatment of patients with relapsed and refractory MM who have failed at least two prior treatments including BTZ [130].

The therapeutic benefits of CFZ-based anti-myeloma therapy were further highlighted in more recent findings from Phase 3 clinical trials. A head-to-head comparison of BTZ-dexamethasone and CFZ-dexamethasone treatments in patients with relapsed and refractory MM showed that patients treated with CFZ-based therapy achieved a nearly two-fold increase in progression-free survival compared to patients treated with BTZ-based therapy (18.7 vs 9.4 months) [113]. Furthermore, the addition of CFZ to the lenalidomide

and dexamethasone combination treatment, a previously designated standard regimen for relapsed and refractory MM, also yielded superior activity in comparison to lenalidomide and dexamethasone without CFZ, as well as combination with BTZ [131]. These findings further validated the therapeutic value of CFZ-based therapies and led to the recent expansion of CFZ indications to include new combination therapies with lenalidomide and dexamethasone as a second-line treatment for patients with relapsed and refractory MM [114].

2.3.5 Advantages Over BTZ

Findings from the Phase 3 clinical trials not only highlighted the importance of CFZ therapy in MM but also suggested CFZ as a superior alterative to BTZ. In addition to having superior efficacy, CFZ also exhibited a substantially more tolerable toxicity profile than BTZ. In particular, incidence of dose-limiting peripheral neuropathy was drastically reduced in CFZ-treated patients compared to those who received BTZ. In an analysis of 136 patients with relapsed and refractory MM, peripheral neuropathy was reported in 15% of the patients receiving CFZ therapy, with only 2% of the patients reporting ≥ Grade 3 peripheral neuropathy [132]. In comparison, BTZ-induced peripheral neuropathy was reported with both higher frequency and increased severity; 35% of BTZ-treated MM patients reported peripheral neuropathy, with 13.4% of the patients exhibiting ≥ grade 3 symptoms [133]. The improved toxicity profile of CFZ has been attributed to its improved selectivity for the proteasome and decreased off-target interactions with the serine protease HtrA2/Omi [117].

The increased tolerability of CFZ treatment has also made it possible to dose CFZ more frequently than BTZ, an important advantage that contributes to the improved anti-myeloma efficacy of CFZ over BTZ. In human xenograft models of MM tumors, CFZ administered on a consecutive-day schedule for up to five days was well-tolerated [6]. Importantly, CFZ delivered on two consecutive days produced better anti-tumor activity compared to BTZ administered according its clinical dosing schedule of once weekly. When proteasome inhibition in the whole blood was assessed, CFZ treatment yielded more sustained proteasome inhibition compared to BTZ treatment. Proteasome activity recovery following CFZ treatment was also much slower compared to BTZ treatment, likely due to

the irreversible nature of CFZ inhibition [6]. Together, these preclinical findings indicated that CFZ may be administered with higher frequency to achieve higher maximal proteasome inhibition without contributing to toxicity.

In line with preclinical findings, clinical assessments also found that twice weekly administration of CFZ was well-tolerated and more efficacious compared to once daily dosing [128]. Importantly, single-agent CFZ delivered on a consecutive two-day schedule was efficacious in BTZ-resistant MM patients and did not exacerbate pre-existing peripheral neuropathy symptoms [11]. As a result, CFZ was approved with a dosing regimen of 20mg/m^2 CFZ administered on days 1, 2, 8, 9, 15, 16, followed by a 12-day rest period for the first cycle, and 27mg/m^2 CFZ for cycles 2 and beyond if tolerated [11, 13, 127]. Since then, further modifications to the CFZ dosing schedule have been evaluated in the clinic. Recently, administration of CFZ as a 30-minute infusion was incorporated into the dosing regimen of CFZ, which allows the dosing of up to 56 mg/m^2 CFZ if tolerated [134]. These modification were based on clinical results which indicated that higher doses of CFZ administered as an infusion over 30 minutes could achieve better anti-myeloma activity and ameliorate side effects compared to the previously established 2-10 minute infusion method [129, 135]. Together, these findings highlight the advantages CFZ has over BTZ in better safety and more flexible dosing, which allows for modifications to achieve further improved therapeutic windows.

2.3.6 Limitations

Both acquired and intrinsic drug resistance are major challenges in CFZ therapy. Single-agent CFZ elicited an overall response of 27% in BTZ-refractory MM patients, with a median duration of response around 7.8 months [11]. These findings indicated that while CFZ remained active in some BTZ-refractory patients, a significant portion of the patients were non-responsive to CFZ therapy and likely had *de novo* resistance to both CFZ and BTZ therapies. Additionally, disease progression following initial response to CFZ is a major threat to the livelihood of these patients. Currently, few effective therapeutic options are available for treating BTZ and/or CFZ resistant MM, and the development of novel strategies to prevent or circumvent CFZ resistance is limited by our lack of understanding regarding the mechanisms responsible for conferring resistance.

In addition to CFZ resistance observed in patients with hematological cancers, overall intrinsic resistance also presents major roadblocks for the expansion of CFZ's therapeutic utility to treat other types of cancers [17]. Due to its potent anti-cancer activity and favorable toxicity profile, CFZ has been actively pursued as a potential treatment for other cancer types including non-hematological malignancies. However, clinical evaluations of CFZ in patients with advanced lung, ovarian, and renal cancers indicated limited efficacy despite CFZ having demonstrated excellent anti-cancer activity in preclinical models of solid cancer. Mechanisms contributing to this discrepancy between clinical and preclinical findings are currently under investigation.

One of the proposed theories accounting for the lack of CFZ activity in solid cancers is its rapid systemic clearance [136, 137]. Clearance of CFZ is primarily mediated by extrahepatic metabolism, where epoxidase and peptidase activities inactivate CFZ by epoxide ring opening and degradation of the peptide backbone [136]. As a result, CFZ has a short half-life of 30 minutes to one hour [17, 138]. In patients with solid tumors, CFZ treatments resulted in little to no anti-tumor efficacy despite substantial proteasome inhibition observed in whole blood cells [17]. The lack of efficacy may be due in part to rapid metabolic inactivation of CFZ, which may hinder the ability of active CFZ to penetrate tumor tissue. To address the metabolic instability of CFZ *in vivo*, a number of recent studies, including one from our group (Chapter 5), investigated the potential of alternative delivery methods aimed to extend the half-life of active CFZ and thereby enhance CFZ efficacy.

2.4 Ixazomib: First Oral Proteasome Inhibitor

Ixazomib (IXA, Ninlaro®) is the first and only oral PI currently approved for the treatment of relapsed and refractory MM. Like BTZ, IXA is an N-capped dipeptidyl boronic acid that preferentially inhibits the β5 subunit of the proteasome reversibly [139]. The boronic acid moiety of IXA is citrate-protected and is readily hydrolyzed upon exposure to aqueous environments, providing it with the improved oral bioavailability compared to BTZ and CFZ [139]. *In vitro*, IXA treatment was found to effectively inhibit cell growth and induce cell death in MM cells. Its activity was also retained in MM cells resistant to BTZ and conventional chemotherapeutics [140]. *In vivo* findings demonstrated that IXA-induced

apoptosis was associated with polyubiquitin accumulation, induction of the UPR, accumulation of pro-apoptotic proteins such as p21, p53, and NOXA, and cleavage of caspases 3, 8, and 9 [140]. Consistent with *in vitro* findings, IXA produced potent anti-cancer activity and prolonged survival in mouse models of MM and lymphoma [140, 141]. Additionally, IXA demonstrated superior tumor penetration and anti-myeloma activity with improved toxicity compared to BTZ [139].

Clinical trial findings indicated that addition of IXA to dexamethasone and lenalidomide combination therapy significantly prolonged progression-free survival of patients with relapsed and/or refractory without increasing toxicities [142]. IXA-treatment resulted in superior response rates in all clinical endpoints measured, with median progression-free survival prolonged by six months compared to placebo groups [143]. Despite sharing the same pharmacophore as BTZ, IXA treatment was associated with much improved toxicity profile. Most cases of peripheral neuropathy observed with IXA treatment were grade 1 or 2 in severity, with grade 3 or 4 peripheral neuropathy observed in 2% of patients, compared to 13.4% observed in BTZ-treatment patients [144]. Based on these clinical observations, IXA received FDA approval in November of 2015 for use in combination with dexamethasone and lenalidomide in MM patients who have received at least one prior therapy [111].

Since IXA was approved very recently, not much is known how its clinical activities compare to those of BTZ and CFZ treatments. For instance, no evidence is available yet to indicate how efficacious IXA is in newly-diagnosed MM patients. Furthermore, there is insufficient clinical data on how IXA performs in BTZ- and CFZ-resistant patients. Limited information is available on the likelihood of cross-resistance between IXA and BTZ and/or CFZ, and no mechanisms of IXA resistance have been reported. Additional clinical trials are currently under way to address these questions which are out of the scope of the current dissertation [111, 145]. The following chapters will discuss PI resistance with a focus on the current understanding of BTZ and CFZ resistance. However, resistance mechanisms reported in BTZ and CFZ models may also be informative about the factors involved in determining cellular sensitivity to other PIs such as IXA.

2.5 BTZ Resistance: Proteasome-Dependent Mechanisms

2.5.1 Mutations in the PSMB5 Gene Encoding β5

In an effort to identify the mechanisms responsible for the development of BTZ resistance, cell line models of acquired PI resistance are commonly used. These models are established by gradually adapting PI-sensitive cancer cells to increasing concentrations of BTZ and are thereafter maintained under selective pressure of BTZ. In such models, the most prevalently identified mechanism accredited to BTZ resistance is the acquisition of point mutations in the *PSMB5* gene encoding the β5 catalytic subunit of the proteasome. Several *PSMB5* mutations have been reported based on cell line models of both hematological and non-hematological cancers [146-149]. Of these, the most commonly observed mutation is a guanine to adenosine change at mRNA position 322 (G322A) of the *PSMB5* gene, which corresponds to an alanine to threonine substitution at amino acid position 49 (Ala49Thr) in the final processed, mature form of the β5 protein [147].

Initially identified in THP-1 human monocytic leukemia cells adapted to BTZ (THP-1/BTZ), the Ala49Thr *PSMB5* mutant was demonstrated by Oerlemans et al. to confer BTZ resistance when introduced into BTZ-sensitive THP-1 cells. A subsequently study by Ri et al. demonstrated a similar link between BTZ resistance and the expression of mutant *PSMB5* by showing that transfection of the Ala49Thr *PSMB5* mutant into BTZ-sensitive KMS-11 cells prevented BTZ-induced polyubiquitin accumulation, G2/M arrest, and apoptotic signaling [148]. The Ala49Thr *PSMB5* mutation was also identified in several cell line models of acquired BTZ resistance, including Jurkat lymphoblastic leukemia cells (JurkatB) [150], KMS-1/BTZ and OPM-2/BTZ MM cells [148], and H460/BTZ non-small cell lung cancer cells [149]. In addition to the Ala49Thr mutation, other mutations were also identified in cell line models of both intrinsic and acquired resistance; these include Ala49Val [146], Ala50Val [146], Met45Val [149, 151], Met45Ile [151], Cys52Phe [149], Cys63Phe [152], and Thr21Ala [151].

All of the reported *PSMB5* mutations are located in the exon 2 region, which encodes for the highly conserved S1 binding pocket of β5 [153]. *In silico* analyses of BTZ bound to yeast proteasome revealed that Ala49 and Thr21 were highly conserved residues and were crucially involved in BTZ binding to β5. Other amino acid residues involved in BTZ binding

inside the S1 binding pocket of the β5 subunit included Ala50, Met45 and Cys52, all of which had been reported as *PSMB5* mutations in association with BTZ resistance. Based on this, it was postulated that mutations in these residues may confer BTZ resistance by interfering with BTZ binding to β5, thereby decreasing the proteasome inhibitory and cytotoxic effects of BTZ in cells [151]. This theory was validated by a more recent study which examined the impact of each *PSMB5* mutation on the proteasome inhibitory and cytotoxic effects of BTZ in yeast [154]. Crystallographic analysis of mutant yeast proteasomes revealed that mutations at the Ala49 position was the most disruptive for BTZ binding to the β5 active site. As well, yeast harboring Ala49 mutations were the most resistant to BTZ-induced proteasome inhibition and cell death. In comparison, the effects of Ala50 and Met45 mutations on BTZ binding and BTZ sensitivity were much less significant [154].

Despite *PMSB5* mutations being the most prevalently reported mechanisms of BTZ resistance in cell line models, none of the reported mutations have been identified in clinical samples to date. Sequencing of MM cells from patients with clinical BTZ resistance failed to identify any mutations in the proteasome catalytic subunits. This suggests that *PSMB5* mutations may not occur as frequently in BTZ-resistant patients as they do in cell line models of resistance, and that expression of proteasome mutations may be a cell line-specific compensatory mechanism to proteasome inhibition [155-158]. Nonetheless, it is important to take such mutations into account for other cell line-based studies of PI activity.

2.5.2 Upregulation of β5

In addition to *PSMB5* mutations, alterations in the expression of the *PSMB5* gene and its encoded β5 protein are the next most frequently reported observation in cell line models of BTZ resistance [146, 147, 149-152, 159, 160]. *PSMB5* upregulation and β5 overexpression was observed in cell line models of intrinsic and acquired BTZ resistance, including the JurkatB lymphocytic leukemia [150], RPMI-8226/BTZ MM [161], and HT-29 colorectal adenocarcinoma cell lines [147, 148, 150-152, 161-164]. Inhibition of β5 induction by siRNA-mediated *PSMB5* knockdown was shown to restore bortezomib sensitivity in several resistant cell lines including the previously mentioned THP-1/BTZ cells [147]. As well, Yang et al. also found that inhibition of the *PSMB5* repressor proteins $G\alpha_{12/13}$ resulted in elevation

of β5 expression and sensitization of Huh7 lung cancer and MiaPaCa2 pancreatic cancer cells to BTZ [162]. These findings suggested a pertinent role for BTZ-induced β5 upregulation in cell survival against BTZ-mediated proteasome inhibition. In line with this, de Wilt et al. and Ri et al. also found that β5 overexpression in BTZ-resistant cells was associated with markedly decreased accumulation of polyubiquitinated proteins when exposed to BTZ concentrations which strongly induced this response in BTZ-sensitive control cells [149]. Additionally, β5-overexpressing cells also exhibited lower levels of pro-apoptotic proteins and caspase activation, effects which were rescued by treatment with higher BTZ concentrations [148, 149].

Interestingly, in cell lines harboring *PSMB5* mutations, *PSMB*5 upregulation did not necessarily correlate with increased protein expression of β5 [147, 151]. Oerlemans et al. reported a drastic 60-fold increase in β5 protein expression in BTZ-resistant THP-1 cells, but PSMB5 expression was found to be only marginally increased [147]. Similarly, Franke et al. described a concentration-dependent upregulation of the *PSMB5* gene in two RPMI-8226/BTZ cell lines with low and high BTZ resistance, but the corresponding β5 expression reflected neither the level mRNA induction nor the extent of BTZ resistance [151]. The authors of the studies pointed out that β5 expression may be regulated post-transcriptionally. However, no further mechanistic explanations were provided regarding such discrepancies in the *PSMB5* and β5 expression levels of BTZ-resistant cells. It is possible that cells harboring *PSMB5* mutations may favor expression of the mutant β5 protein over the wildtype as a survival mechanism. Further investigation would be necessary to determine whether regulation of the expression of mutant β5 expression differs from that of wildtype β5. Additionally, Orelemans et al. also noted that drastic upregulation of β5 in THP-1/BTZ cells did not yield similar increases in β5 activity levels [147]. However, since mutations in the *PSMB5* gene has been shown to interfere with the binding of BTZ to the β5 active site, it is possible that the mutations may also affect the binding of the proteasome substrate in this case.

The findings summarized here provide evidence for β5 upregulation as a potential compensatory mechanism to maintain proteasome activity in the presence of BTZ, and

thereby prevents apoptosis. While upregulation of β5 remains one of the most commonly observed PI-induced alterations in proteasome expression, it is unclear whether such alterations in the expression of *PSMB5* and β5 are driving factors of BTZ resistance or part of a feedback response to prolonged proteasome inhibition. Further investigation of the functional impact of β5 upregulation in the context of BTZ resistance are necessary to tease out its role in determining BTZ sensitivity. Additionally, it is important to take note that BTZ-induced β5 upregulation may include mutant forms of β5. Since it is currently unknown whether *PSMB5* mutations and β5 overexpression confer BTZ resistance independent of one another, further investigations addressing this question are warranted.

2.5.3 Increased Expression and Activities of β1 and β2

Upregulation of β1 and β2 have been reported in both hematological and solid cancer cell line models of acquired BTZ resistance, such as lung cancer (H460/BTZ and A549/BTZ), myeloid leukemia (HL-60a), monocytic leukemia (THP-1/BTZ), and MM (RPMI-8226/BTZ) cells [147-149, 151, 165]. Compared to β5, much less is known about the roles of β1 and β2 in determining cellular response to BTZ. Previous findings suggested that activities of the β1 and β2 subunits may be important contributors to cell viability and the overall cellular response to proteotoxic stress [166, 167]. For example, Chondrogiani et al. showed that WI38 fibroblast cells stably overexpressing β1 had better proliferating capabilities and augmented capacity to cope with oxidative stress compared to control cells [166]. Furthermore, Heinemeyer et al. demonstrated that knockdown of β5 in yeast cells was not sufficient to induce cell death, and that inhibition of either β1 or β2 in addition to β5 was required to commit cells to apoptosis [167]. These findings challenged the previous notion that β5 activity is the most important proteasome activity in mediating cell survival and suggested that activities of β1 and/or β2 may also play an important role in PI-mediated cytotoxicity. However, due to the lack of β1- and β2-selective substrates at the time, no further mechanistic studies were done to examine the direct relationships between the activities of the β1 and β2 subunits and cellular response to PIs.

Consistent with earlier findings, a more recent study by Britton et al. demonstrated using subunit-selective proteasome inhibitors that inhibition of the β5 subunit alone was not

sufficient to achieve complete cell death, and that simultaneous inhibition of additional proteasome subunits was required to achieve maximal cytotoxicity [168]. The authors found that co-inhibition of the β1 subunit sensitized MM cells to the β5-targeting proteasome inhibitor NC-005, which suggested that co-targeting of β1 could enhance the activity of β5-targeting PIs such as BTZ [168]. A subsequent study published last year further explored the effect of co-targeting of β2 in the context of BTZ resistance and found that selective inhibition of β2 could restore BTZ sensitivity in resistant AMO-1a MM cells [169]. Together, these findings supported the upregulation of non-β5 activities as a potential survival mechanism against BTZ-induced cytotoxicity and provided initial evidence for the potential of co-targeting non-β5 subunits as a strategy to overcome resistance.

2.5.4 Downregulation of IP Catalytic Subunits

Downregulation of IP expression has been implicated in both intrinsic and acquired BTZ resistance models [149, 151, 169-171]. Low IP expression was found to be associated with intrinsic BTZ resistance in both hematological and non-hematological cancer cells [169, 170, 172]. In particular, Busse et al. compared IP expression and BTZ sensitivity in 12 solid cancer and 12 hematological cancer cell lines and found that solid cancer cells across the board displayed lower levels of IP catalytic subunits and lower BTZ sensitivity compared to hematological cancer cells [170]. These findings indicated that inherent differences in IP expressions may contribute to the differential BTZ sensitivities among hematological and solid cancer cell lines. In addition to relative IP expression, the ratio of IP and CP levels was also shown to differ in cell lines with varying BTZ sensitivity. Kraus et al. found that concurrent IP upregulation and CP downregulation induced by IFN-γ treatment sensitized cells to BTZ [170].

Consistent with findings from intrinsic resistance models, IP downregulation was also found to contribute to acquired BTZ resistance. Niewerth et al. reported downregulation of IP catalytic subunits along with upregulation of CP catalytic subunits in all three BTZ-resistant MM and lymphoma cell lines (RPMI-8226/BTZ, THP-1/BTZ, and CEM/BTZ) compared to respective parental controls [171]. These changes in proteasome composition

were also accompanied by increased total proteasome content. This is consistent with findings in intrinsic resistance models and support the notion that overexpression of the proteasome as a whole, in addition to higher IP/CP ratios, may contribute to acquired BTZ resistance. IFN-γ-induced IP upregulation rescued BTZ sensitivity in the resistant cells, evident by increased accumulation of polyubiquitinated proteins and PARP cleavage following BTZ exposure [171]. High CP and low IP expression patterns have also been associated with decreased BTZ sensitivity in the clinic [163, 172, 173] [174]. Lu et al. reported higher β5 expression levels in MM cells isolated from BTZ-refractory patients compared to cells from BTZ-sensitive patients [163]. Similarly, low expression of IP was associated with BTZ resistance in MM patients, whereas high IP/CP ratios were correlated with better BTZ response [172, 173].

These findings suggested that cells inherently expressing high levels of IP and low levels of CP may be more susceptible to BTZ-induced cell death [170]. Upregulation of IP expression in response to long-term BTZ treatment may provide cells with survival advantages against proteasome inhibition. Conversely, low baseline expression of IP may also be a predictor for poor BTZ response and a contributing factor to intrinsic resistance to BTZ.

2.5.5 Upregulation of Non-Catalytic Proteasome Subunits

Upregulation in non-catalytic proteasome subunits have been observed in BTZ resistance cell line models in addition to alterations in the expression of catalytic subunits [149, 151, 160]. The proteasome structural subunit α7 was found to be upregulated along with β5 overexpression in RPMI-8226/BTZ, A549/BTZ, and H460/BTZ cells with acquired resistance [149, 151]. Similarly, BTZ-adapted Namawa^{ad} cells also displayed increased levels of the proteasome core subunits α3, β4, and β6 in concurrence with overexpression of all three constitutive β-subunits (β1, β2, β5) [160]. These findings suggest that upregulation of the constitutive proteasome complex as a whole may be an important compensatory mechanism for cells to overcome BTZ-induced insult. In line with this, Fuchs et al. found that BTZ-resistant cells harbored higher levels of the proteasome maturation protein (POMP), further indicating that *de novo* proteasome synthesis may be increased in cells as a protective response to BTZ treatment [160].

2.5.6 Increased Proteasome Transcription and Assembly

Related to the overexpression of proteasome in BTZ-resistant cells is the reported upregulation of the transcription factor Nrf2 (Nuclear factor-erythroid 2-like 2) and the proteasome assembly chaperone protein POMP [175-177]. Li et al. reported enhanced levels of POMP in a panel of four MM cell lines with acquired BTZ resistance [175]. Expression of POMP was shown to be required for both conferring and maintaining BTZ resistance in OPM-2 and CAS-6/1 MM cells. Additionally, the transcription factor Nrf2, which binds to the promoter of POMP, was found to be upregulated in both BTZ-resistant cells and BTZ-naïve cells challenged with BTZ short-term. Overexpression of Nrf2 resulted in upregulation of POMP and increased CT-L proteasome activity, whereas inhibition of Nrf2 activity sensitized resistant cells to BTZ. These findings led the authors to conclude that POMP upregulation via increased Nrf2 transcriptional activity contributes to BTZ resistance in MM cells, and proposed that targeting Nrf2 function may restore BTZ sensitivity [175]. In support of this proposed theory, the same group later demonstrated that Nrf2 inhibitor ATRA (all-trans retinoic acid) could indeed sensitize primary myeloma tumor cells to BTZ [177]. Rushworth et al. also found an association with Nrf2 upregulation and BTZ resistance in AML217, AML306, and THP1 cells. In this case, the authors suggested that BTZ-induced Nrf2 upregulation protected cells from apoptosis by reducing reactive oxygen species levels [176]. Collectively, these findings indicate an important role for Nrf2 in determining BTZ response in cells and support the targeting of Nrf2 as a potential strategy to enhance BTZ sensitivity.

2.5.7 Summary and Implications

Of the proteasome-dependent mechanisms of BTZ resistance reported so far, mutations in the *PSMB5* gene and β5 overexpression are the most prevalently observed across cell line models. However, *PSMB5* mutations have not been identified in the clinic so far, suggesting that resistance driven by the development of such mutations may be not have major impact beyond *in vitro* settings. On the other hand, alterations in proteasome expression and activities have been observed in BTZ-resistant patients. In particular, BTZ-induced overexpression of CP catalytic subunits present potential targets for therapeutic intervention. To this end, co-targeting of β1 or β2 has shown promising activity in enhancing the cytotoxic effects of BTZ and other β5-targeting agents. For instance, findings

by our group have demonstrated the potential of co-targeting β1 as a potential strategy to enhance BTZ activity in resistant cells (chapter 4 of this dissertation work). Such preliminary findings provide important insights into the potential utility of β1- and β2-targeting PIs and justify the development of next-generation PIs with selectivity toward activities other than the CT-L activity.

2.6 BTZ Resistance: The Unfolded Protein Response Pathway

2.6.1 The UPR & PI-Induced Cytotoxicity

Alterations in the unfolded protein response pathway (UPR) have been found to be especially impactful for BTZ response in MM and other hematological cancers. MM cells are characterized by massive antibody secretion and are equipped with an expansive and developed endoplasmic reticulum (ER) network [75]. Due to their high demands for protein secretion, MM cells are prone to ER stress caused by accumulation of misfolded and unfolded proteins, defective protein trafficking, and impairment in protein degradation. Thus, MM cells are exceptionally sensitive to the disruption of cellular processes involved in maintaining ER homeostasis [178].

ER stress triggers the activation of a set of signaling pathways called the UPR, which attempts to resolve the stress by halting protein synthesis, inducing transcription of stress response proteins, and eliminating misfolded and damaged proteins from the ER [179, 180]. UPR activation is initially pro-survival, as immediate downstream signaling attempts to maintain protein homeostasis [181]. However, prolonged ER stress eventually results in UPR-triggered apoptosis, known as terminal UPR, via induction of the transcription factor CHOP [182]. Because pro-survival UPR signaling requires proteasome function to carry out downstream ER-associated degradation of misfolded proteins, it is now well-known that proteasome inhibition is a major trigger for terminal UPR [75].

2.6.2 Xbp-1 Downregulation

Since the UPR is a major mechanism through which BTZ induces cell death, it is unsurprising that one of the key transcription factors involved in mediating UPR signaling, the X-box binding protein 1 (Xbp1), was found to have an important role for determining BTZ sensitivity in several hematological cell lines and patient-derived tumors [183, 184].

Xbp1 function is particularly important for MM pathogenesis; activation of Xbp1 signaling is crucial for driving plasma cell differentiation and maintaining ER capacity to cope with cellular stress associated with high levels of immunoglobulin production [179]. Xbp1 downregulation has been associated with BTZ resistance in MM and other cancer cells derived from B-cell malignancies. The proposed mechanism underlying Xbp1 downregulation and BTZ resistance involves alterations to the B-cell differentiation program that drive cells toward a phenotype characterized by lower protein production and ER stress, making cells less sensitive to BTZ-induced proteotoxicity. Findings supporting this proposed mechanism are further discussed below.

The role of Xbp1 in determining BTZ response was investigated by several groups in both MM cell lines and primary MM samples [183-185]. Ling et al. reported a strong inverse relationship between BTZ IC_{50} and total Xbp1 mRNA in a panel of MM cells lines. Low Xbp1 expression was detected in conjunction with decreased expression of other UPR regulator proteins and low levels of immunoglobulin production in BTZ-adapted MM cells and MM tumors with clinical resistance [183]. Similarly, decreased ATF6 expression and ER volumes were also observed in BTZ-resistant MM cells and patient samples, and high Xbp1 expression was correlated with better outcome in BTZ-treated MM patients. [184, 185]. In general, low Xbp1 expression has been associated with decreased BTZ sensitivity across the board. These findings suggest that MM cells with lower protein workload and UPR activation may have a survival advantage against BTZ-induced cytotoxicity. However, these studies did not offer any potential mechanisms through which Xbp1 downregulation may mediated BTZ resistance [184].

2.6.3 Xbp1 and Plasma Cell Differentiation

Leung-Hagesteijn et al. further explored the role of Xbp1 expression and function in determining the BTZ response of MM cells [173]. In contrast to Ling et al., who concluded that decreased Xbp1 was a surrogate marker for BTZ resistance rather than a determining factor, Leung-Hagasteijn reported that loss of Xbp1 in fact did confer BTZ resistance in MM cell lines [173]. In addition, Leung-Hagasteijn et al. also identified two Xbp1 mutations in BTZ-resistant MM tumors, Xbp1-L167I and Xbp1s-P326R, which were found to prevent Xbp1 activation and affect Xbp1 tertiary structure [173, 186]. Constitutive expression of

these Xbp1 mutant transcripts were found to be functionally inhibiting and resistance-conferring [173]. BTZ-refractory MM tumors harboring these Xbp-1 mutations also displayed downregulation of plasma cell maturation genes, along with lower Xbp1 expression, lower UPR activation, decreased expression of plasma cell surface markers, and reduced immunoglobulin synthesis, which were indicative of a less differentiated B-cell phenotype [173].

Based on these observations, Leung-Hagesteijn et al. postulated that MM cells undergoing maturation stages between activated B cells and pre-plasmablasts may be less sensitive to BTZ. This "sweet spot" in plasma cell differentiation was characterized by significantly reduced ER burden compared to fully differentiated plasma cells, which would make cells less sensitive to proteotoxic stress and more resistant to PI-induced cytotoxicity [173]. This theory was based on previous reports which indicated a crucial role for Xbp1 in governing crucial events in plasma cell differentiation such as immunoglobulin secretion and ER remodeling [187]. Additionally, this proposed mechanism by which Xbp1 downregulation de-regulates plasma cell differentiation confers resistance was supported by another study by Perez-Galan et al., which showed that promotion of B cell maturation toward the plasmablast differentiation stage could mediate BTZ resistance in mantle-cell lymphoma cells [164]. The authors of this study noted a decrease in Xbp-1 activation in cell lines with intrinsic BTZ resistance as well as those with acquired resistance, however the mutational status of Xbp-1 was not examined in this case [164].

2.6.3 Summary & Implications

The findings summarized here indicate a potential role for Xbp1-mediated reprogramming of B-cell differentiation in conferring BTZ resistance in B-cell malignancies [188]. Conclusions from these investigations also confirm the previously proposed notion that cells with higher proteasome workload in general are more sensitive to the cytotoxic effects of PIs [189-192]. Based on these findings, it may be advantageous to explore strategies that better target malignant B-cells expressing specific differentiation markers. Such strategies may be able to achieve better anti-cancer efficacy and potentially reduce toxicities associated with BTZ-induced apoptosis of non-malignant cells [193]. Previous findings have also suggested that BTZ-resistant cells with downregulated UPR function may be more

susceptible to cell death triggered by specific inhibition of the UPR pathway [194]. Based on this, selective targeting of BTZ resistant cells exhibiting low UPR function with other UPR-targeting chemotherapeutics may be an effective strategy to circumvent resistance. Currently, it is unknown whether Xbp1-mediated resistance involves other UPR mechanisms outside of the B-cell differentiation program. For instance, increased protein workload and higher reliance on protein processing pathways have been proposed as a potential reason underlying the differential PI sensitivity of hematological and solid cancer cells to proteasome inhibition [195]. However, the mechanisms involved in mediating the differences in sensitivity have not been elucidated. In this regard, the role of the UPR in determining BTZ sensitivity in solid cancer cells may be worthwhile to investigate.

2.7 BTZ Resistance: The Stress Response Pathway

2.7.1 Heat Shock Proteins

The heat shock response (HSR) pathway is another stress-response pathway triggered by ER stress such as that caused by proteasome inhibition. This pathway is highly conserved among eukaryotic cells and is an important part of cellular repair mechanisms against stress-induced damages [196]. HSR is triggered upon the detection of aggregated misfolded or unfolded proteins inside the cytosol [197], which induces the transcription of heat shock proteins such as Hsp70 and Hsp90 and other stress-related chaperone proteins including BiP/GRP78 [198, 199]. This process aims to re-establish protein homeostasis by preventing damaged proteins from aggregating and facilitating the unfolding and refolding of aggregated proteins and misfolded proteins [200]. These events serve as a coping mechanism for cells to tolerate the short-term proteotoxic stress caused by factors such as proteasome inhibition. A number of studies have suggested that cells may hijack this HSR-mediated coping response to gain survival advantages against proteasome inhibition. Overall, increased expression of HSR-related proteins is recognized as a pro-survival mechanism and has been association with both intrinsic and acquired BTZ resistance [201]. Additionally, interference of the HSR pathway has also demonstrated BTZ-sensitizing effects, suggesting that the HSR pathway may serve as a potential therapeutic target for BTZ resistance [201].

Several members of the heat shock response pathway have been implicated in cell line models of BTZ resistance [202-205]. Gene expression profiling of diffuse large B-cell

lymphoma cells revealed higher expression levels of the chaperone proteins Hsp27, Hsp70, and Hsp90 in SUDHL-4 cells with intrinsic BTZ resistance compared to the BTZ-sensitive SUDHL-6 cells [202]. Increased Hsp27 expression was shown to be associated with BTZ resistance based on the opposing effects of Hsp27 overexpression and knockdown on cellular sensitivity to BTZ [203]. The exact role of Hsp27 in BTZ resistance remains unknown; it was speculated that interactions between Hsp27 and regulators of the apoptotic pathway may contribute to its protective role against PI cytotoxicity.

Hsp70 expression was associated with BTZ resistance in lymphoma cells and co-treatment of a Hsp70 inhibitor was found to potentiate the BTZ activity in melanoma cells [202, 206]. In contrast, amplification of a negative regulator of Hsp70 was detected in BTZ-resistant lung cancer cells, which suggested that Hsp70 downregulation may be pro-survival against BTZ in this case [152]. These findings suggest that Hsp70 may have cell line-specific roles in determining BTZ sensitivity. However, since the authors did not further confirm a functional role of Hsp70 in conferring BTZ resistance, it is also possible that downregulation of Hsp70 may be an artifact of larger-scale chromosomal changes induced by long-term exposure to BTZ. More detailed assessments of the functional impact of Hsp70 regulation in the context of BTZ resistance would be necessary to confirm whether such alterations observed in Hsp70 expression have direct relevance in determining BTZ sensitivity in cells.

2.7.2 BiP/GRP78 and the Hsp90 Chaperone Compelx

Both the ER chaperone protein BiP/GRP78 and the molecular chaperone protein Hsp90 have been reported to have cytoprotective and resistance-conferring roles in against BTZ-induced cell death [207, 208]. High BiP/GRP78 expression was associated with poor BTZ response in mantle cell lymphoma and diffuse large B-cell lymphoma (DLBCL) patients [207, 208] whereas BiP/GRP78 knockdown sensitized DLBCL cells to BTZ-induced cytotoxicity. BiP/GRP78 was also found to accumulate with the Hsp90 chaperone complex in BTZ-resistant mantle cell lymphoma cells [207]. The interaction between BiP/GRP78 and Hsp90 proved to be essential for BTZ resistance, as disrupting the formation of this interaction using an Hsp90 inhibitor resulted in inhibition of the UPR and sensitized cells to BTZ [207]. Based on these findings, the authors proposed that stabilization of BiP/GRP78 by Hsp90 could mediate BTZ resistance by increasing pro-survival UPR activity and mitigating ER burden [207].

Inhibition of Hsp90 has shown promising BTZ-sensitizing activities both *in vitro* and clinically [207, 209-213]. The small molecule Hsp90 inhibitors IPI-504 and 17-AAG (Tanespimycin) were both demonstrated to effectively restore BTZ sensitivity in BTZ-resistant mantle cell lymphoma and MM cells [207, 209]. Clinically, 17-AAG was also found to produce robust anti-myeloma activity when used in combination with BTZ in both pretreated and BTZ-refractory MM patients [210]. In addition to hematological cancers, other Hsp90 inhibitors have also demonstrated BTZ-potentiating effects in solid cancers including Ewing sarcoma and breast cancer [211-213]. Together, these findings provide evidence for the targeting of Hsp90 as a potentially effective strategy to improve BTZ efficacy and overcome BTZ resistance.

2.7.3 Summary & Implications

Findings from these studies indicate that components of the HSR pathway play important roles in mediating BTZ-induced cell death and serve as promising potential targets in BTZ-resistant cancer cells. Overall, upregulation of the HSR proteins is associated with cancer cell survival against proteasome inhibition. This is expected as the HSR proteins play crucial roles in maintaining ER homeostasis and protecting the cells from proteotoxic stress. Due to the diverse client protein population regulated by the chaperone activities of the HSR, inhibition of HSR proteins may affect a wide variety of cellular processes including those involved in regulating growth, survival, and apoptosis. Currently, several small molecule inhibitors targeting HSR proteins are under clinical development as anti-cancer agents [213]. In this regard, co-inhibition of HSR components and the proteasome has shown promise as a combined treatment to enhance the anti-cancer activity of BTZ. Further validation of this strategy is underway and may provide important insights into potential strategies to improve PI efficacy in both hematological and solid malignancies [214]. Additionally, it would also be important to evaluate the role of the HSR in resistance of other PI agents and assess whether HSR inhibition may be utilized as a general strategy to enhance PI activity.

2.8 BTZ Resistance: The Apoptotic Signaling Pathway

The Bcl-2 family is divided into three subfamilies and consists of both pro-apoptotic and anti-apoptotic regulator proteins. Pro-apoptotic regulators such as Bax and Bim activate apoptosis by inserting into the mitochondrial membrane upon detecting death signals and facilitating the release of pro-apoptotic protein cytochrome c from the mitochondria [215]. Conversely, anti-apoptotic regulators such as Bcl-2, Bcl-x_L, and Mcl-1 prevent mitochondrial permeabilization by interacting with pro-apoptotic regulators through BH-domains [216]. Unlike the first two subfamilies of Bcl-2 family proteins, which contain multiple BH-domains, the last subfamily consists of only BH3 domains and are thus named after this structural distinction. The BH3-only subfamily contains proteins such as Noxa, PUMA, and BIM, and act as sensors of cellular stresses and can facilitate apoptosis when activated [217]. Several studies have demonstrated the importance of Bcl-2 family members in mediating BTZ-induced cell death and resistance [164, 218-222]. Of the Bcl-2 family members implicated, the BH3-only protein Noxa plays a central role in conferring BTZ resistance through various interactions with both pro- and anti-apoptotic regulators [73, 76, 218, 223].

2.8.1 Noxa

Interactions between the BH3-only protein Noxa and the anti-apoptotic protein Mcl-1 have been shown to be crucial in mediating BTZ-induced apoptosis [73, 76, 223, 224]. In particular, induction of Noxa, but not of other BH3-only proteins, is prevalently observed in cell lines treated with BTZ and is considered an important regulator of BTZ-induced cell death [76]. Disruption of Noxa upregulation by genetic silencing was found to desensitize mantle cell lymphoma cells to BTZ, suggesting that Noxa expression is crucial for mediating BTZ-induced cytotoxicity [76]. In line with this, Gomez-Bougie et al. found that apoptosis in BTZ-treated MM cells was highly dependent on both Noxa-induction and Mcl-1 expression. Expression levels of Mcl-1 and Noxa were found to have counteracting effects of BTZ-induced apoptosis: whereas downregulation of Mcl-1 sensitized MM cells to BTZ, Noxa knockdown rendered cells BTZ resistant. Noxa upregulation in BTZ-treated MM cells was also detected in conjunction with decreased complexation of Mcl-1 with the pro-apoptotic proteins Bak and Bim and increased mitochondrial permeabilization, suggesting that increased expression of Noxa had disruptive effects on the partnering of pro-apoptotic proteins with Mcl-1. Based on these observations, it was postulated that BTZ-induced cell

death in MM cells is mediated by an increase in interactions between Noxa and Mcl-1, which in turn displaces Mcl-1 binding to pro-apoptotic proteins and thus enhances apoptosis activation [223]. In support of this theory, other studies have also confirmed Noxa upregulation and Noxa-Mcl-1 interactions as key modulators of BTZ-induced apoptosis in cells [73, 76, 224].

Since Noxa plays an indispensable role in BTZ-induced cell death, it is unsurprising that downregulation of Noxa has been proposed to be a potential mechanism of BTZ resistance. An early study by Rizzatti et al. showed that Noxa knockdown in mantle cell lymphoma cells significantly decreased cell death following BTZ treatment [76]. More recently, Leshchenko et al. suggested that epigenetic changes resulting in Noxa downregulation may play a role in conferring BTZ resistance in mantle cell lymphoma cells [222]. Demethylation of the Noxa promoter by the hypomethylating agent decitabine was associated with induction of Noxa, increase apoptosis, and sensitization to BTZ. Furthermore, combined treatment of decitabine and BTZ lead to synergistic anti-cancer activities in both BTZ-resistant cell lines and xenograft models. Together, these findings further demonstrated the importance of Noxa in determining BTZ sensitivity in mantle cell lymphoma cells. These findings justified the targeting of Noxa, either by chemical inhibition or through epigenetic priming (i.e. methylation inhibition), as a potentially effective strategy to enhance BTZ efficacy [222].

2.8.2 Mcl-1

Mcl-1 overexpression has also been found to contribute to BTZ resistance both *in vitro* and in the clinic [225-227]. BTZ-induced Mcl-1 upregulation was reported in both hematological and solid cancer cells and was viewed in general as cytoprotective against BTZ-induced cytotoxicity [73, 223, 225, 227, 228]. Increased expression of Mcl-1 was reported in MM and Jurkat lymphoma cells in association with BTZ resistance, with Mcl-1 upregulation in MM cells attributed to UPR activation and increased transcription of Mcl-1 [225, 227]. Similar association between high Mcl-1 expression and BTZ resistance was also observed under clinical settings. Wuilletme-Toumi et al. reported that patients who expressed high levels of Mcl-1 were associated with poor BTZ response and higher risk of disease relapse compared to patients with lower baseline Mcl-1 expression [226]. These results suggest that Mcl-1 may be a useful predictive marker for clinical BTZ response. In line with these findings, Mcl-

1 inhibition has been proposed by several studies as a potentially effective strategy to combat Mcl-1-mediated BTZ resistance. Knockdown of Mcl-1 activity has been shown to sensitize cells to BTZ in MM, acute T-cell leukemia, and glioma cell line models [223, 227, 228]. Additionally, targeting of the MEK1/2 pathway, which regulates interactions between Mcl-1 and pro-apoptotic proteins, has also been suggested to improve BTZ efficacy in resistant MM cells [229].

2.8.3 Bcl-2

Smith et al. recently suggested that binding of Bcl-2 to Noxa contributed to BTZ resistance in Jurkat lymphoid cells by blocking interactions between Noxa and other anti-apoptotic proteins, thereby preventing BT-induced apoptosis [218]. Bcl-2 and Noxa expression had opposing effects on BTZ sensitivity: whereas genetic silencing of Bcl-2 sensitized Jurkat cells to BTZ, Noxa knockdown rendered the cells BTZ resistant. Introduction of Bcl-2 in Jurkat cells was cytoprotective against BTZ, with the lymphoid-associated Bcl-2 mutant with the highest Noxa binding affinity achieving the most cytoprotective effects [218]. Although the study did not provide evidence for how BTZ resistance mediated by Bcl-2 may be connected to Mcl-1-based mechanisms, Smith et al. speculated that Bcl-2 may serve as a lower affinity, higher capacity neutralizer of Noxa that interferes with interactions between Noxa and Mcl-1. Based on this, it is likely that upregulation of Bcl-2 protects cells from BTZ-induced cell death by antagonizing the pro-apoptotic functions of Noxa and Mcl-1, thereby conferring resistance. Additionally, Bcl-2 inhibition has demonstrated BTZ-sensitizing effects in mantle cell lymphoma, diffuse large B cell lymphoma, and melanoma cell lines [224, 230, 231]. As well, high Bcl-2 expression was also proposed as a contributing mechanism to intrinsic BTZ resistance in melanoma cells [231, 232].

2.8.4 Summary

These findings discussed here suggest that alterations in the expressions and interaction of Bcl-2 family proteins play an important role in determining BTZ sensitivity in cells. Both Mcl-1 and Bcl-2 upregulation were shown to be cytoprotective and resistance-conferring against BTZ-induced cytotoxicity. Interestingly, central to the proposed resistance-conferring mechanisms of Mcl-1 and Bcl-2 was the sequestration of Noxa. Overall, decreased Noxa activity is associated with decreased apoptosis activation by BTZ and

desensitization of cells to BTZ. Inhibition of Noxa activity may be mediated through increased interactions with Mcl-1 or Bcl-2, as well as epigenetic downregulation of Noxa expression. Several BTZ-sensitizing strategies have been proposed based on the findings discussed here. Leshchenko et al. suggested epigenetic priming with the hypomethyating agent decitabine as a potential strategy to overcome BTZ resistance in mantle cell lymphoma cells [222]. In this regard, decitabine and its predecessor 5-azacytidine have demonstrated promising clinical activities against acute myeloid leukemia and myelodysplastic syndrome, respectively [233, 234]. However, despite such agents having promising anti-cancer activities alone, evidence supporting the combination use of decitabine and BTZ is generally lacking. Thus, additional assessments are needed to determine the efficacy and safety of hypomethylating agents in combination with BTZ in resistant patients. Additionally, the targeting of Bcl-2 and Mcl-1 have also been proposed as potential strategies to overcome BTZ resistance. Further validation of the BTZ-potentiating activities of such strategies are also warranted.

2.9 Carfilzomib Resistance

In comparison to BTZ resistance, much less is known about the mechanisms contributing to CFZ resistance, likely due to its shorter clinical experience. Of the mechanisms proposed to date, upregulation of the drug efflux transporter P-glycoprotein is the most commonly reported mechanism of acquired CFZ resistance, and was also demonstrated to mediate resistance to other epoxyketone-containing PIs but not to BTZ. Other proposed mechanisms of CFZ resistance include alterations in the autophagy-lysosome pathway and novel interactions between mutant p53 and Nrf2. These mechanisms are not as well-validated across studies as P-gp upregulation and their impact on BTZ sensitivity is unclear. The following sections summarize the current understanding of potential mechanisms driving CFZ resistance.

2.9.1 P-glycoprotein Upregulation

P-glycoprotein (P-gp) is a transmembrane protein that functions in an ATP-dependent manner to extrude a wide variety of xenobiotic substrates out of the cell [235]. Its activity has been attributed to cancer resistance to a broad spectrum of chemotherapeutics including doxorubicin, paclitaxel, and vincristine [236-238]. Initial clues of a role for P-gp in

mediating CFZ resistance came from an earlier study by Gutman et al., which reported P-gp overexpression in KMS-11 cells with acquired resistance to epoxomicin, an early-generation epoxyketone-based PI and the predecessor of CFZ [8, 239]. Resistant KMS-11 cells showed rapid upregulation of P-gp expression when challenged with epoxomicin, and P-gp inhibition rescued the apoptotic response of KMS-11R cells to epoxomicin [239]. These results indicated that upregulation of P-gp was the major mediator of resistance against epoxomicin-induced cell death. KMS-11/R cells did not exhibit any cross resistance to BTZ and P-gp inhibition had no significant impact on BTZ sensitivity in these cells. Based on these findings, it is likely that P-gp-mediated resistance is selective for epoxyketone-based PIs and does not have a major effect on BTZ sensitivity [239].

Since CFZ shares a great deal of structural similarities to epoxomicin, it was speculated that CFZ activity may also be susceptible to P-gp-mediated resistance [239]. In line with this, CFZ cross-resistance was observed in doxorubicin-resistant RPMI-8226 MM cells [125, 240] and vinblastine-resistant CEM lymphoid cells [241], both of which harbored P-gp overexpression. P-gp inhibition rescued CFZ sensitivity in both cell lines, albeit to varying degrees, indicating that CFZ cross-resistance was mediated at least in part by P-gp function. Our study (chapter 3 in this dissertation work) confirmed P-gp as the major mediator of CFZ resistance in H23/CFZRR lung and DLD-1CFZRR colon adenocarcinoma cell line models [242]. We found that P-gp, but not other drug efflux transporters, was upregulated in H23/CFZRR and DLD-1CFZRR cells, and that P-gp inhibition markedly reversed CFZ resistance in both cell lines [242]. Consistent with our findings, Muz et al. also reported P-gp upregulation as a major contributor of CFZ resistance in MM cells [243]. In this case, activation of the hypoxia response pathway was found to be responsible for the induction of P-gp expression, and CFZ sensitivity could be restored by P-gp or HIF-1 inhibition (key regulator of the hypoxia response pathway) [243].

P-gp-mediated resistance was also reported for several other PIs sharing structural similarities with CFZ and epoxomicin [241, 242]. Cross-resistance was found between CFZ and the β1/1i-selective inhibitor YU-101 and IP inhibitors ONX-0912 and ONX-0914 in CFZ-resistant lung and colon cancer cells overexpressing P-gp [241, 242]. As noted before, cross-resistance to BTZ was found to be marginal in all cases. This selectivity of P-gp transport for

epoxyketone-based PIs provides an interesting distinction of P-gp-mediated resistance to different PIs and can potentially serve as an important predictor of how cells may respond to different classes of PI agents. Furthermore, as we gain a better understanding of the mechanisms responsible for PI resistance in the clinic, this information may be useful in predicting whether patients with resistance to certain classes of PI agents will remain responsive to others PI agents.

Although the role of P-gp in CFZ resistance has been demonstrated in a number of cell line models [240-242, 244], clinical evidence of P-gp-mediated resistance has been scarce. Using gene expression analysis of tumor cells obtained from MM patients, Hawley et al. showed that upregulation of the P-gp-encoding *ABCB1* gene was associated with poor disease prognosis [240]. More recently, Soriano et al. reported a nearly 4-fold increase in *ABCB1* expression in plasma cells isolated from a CFZ-refractory MM patient compared to cells isolated before disease progression [245]. This is the first and only evidence thus far demonstrating a potential role of P-gp upregulation in clinical CFZ resistance. Further validations with larger sample sizes are necessary to confirm whether this is a clinically important mechanism of CFZ resistance.

2.9.2 Induction of Cytoprotective Autophagy

The autophagy-lysosome pathway is a major protein degradation pathway responsible for the destruction and recycling of long-lived proteins, damaged organelles, and non-functional proteins such as those produced during biosynthesis [246]. Autophagy is characterized by the capturing of substrates inside membrane vesicles called autophagosomes that fuse with lysosomes, where protein contents are digested [247]. This process normally occurs at low basal levels and is important for maintaining overall protein and energy homeostasis. Although autophagy was originally believed to be a parallel but distinct proteolytic pathway to the UPS, recent evidence suggests that cross-talk between the two pathways may occur, especially under stress-induced settings [248]. Of interest, autophagy has been shown to readily activate in cells exposed to PIs—a compensatory mechanism suggested to be cytoprotective of cells against proteotoxic stress resulted from inhibition [249, 250]. In line with this, autophagy inhibition has been shown to increase cellular sensitivity to BTZ in both BTZ-naïve and BTZ-adapted cells, further indicating the

importance of autophagy in determining PI sensitivity.

A strong correlation between acquired CFZ resistance and upregulation of the pluripotency reprogramming factor Kruppel-like factor 4 (KLF4) was reported by Riz et al. in CFZ-resistant MM cells (KMS-11/Cfz and KMS-34/Cfz) [251, 252]. Both resistant cell lines exhibited increased levels of KLF4 at the mRNA and protein levels, and its overexpression was associated with decreased CFZ sensitivity [252]. Both KMS-11/Cfz and KMS-45/Cfz cells displayed KFL-4 upregulation despite having different P-gp expression patterns, which suggested that KFL4 upregulation may mediate CFZ resistance regardless of P-gp upregulation. In conjunction with KFL4 upregulation, both resistant cell lines also exhibited increased levels of the autophagy-related gene sequestosome 1/p62 (SQSTM1/p62), a known transcriptional target of KFL4 and a crucial adaptor protein involved in shuttling polyubiquitinated proteins to the UPS and the autophagy-lysosome pathway. SQSTM1 upregulation in the CFZ-resistant cells was associated with enhanced autophagic activity, characterized by increased stabilization of autophagosome structures and autophagic flux. Furthermore, disruption of the autophagy-lysosome pathway by chloroquine treatment sensitized both KM-11/Cfz and KMS-34/Cfz cells to CFZ. Based on these observations, it was postulated that SQSTM1-mediated induction of autophagy protected CFZ-resistant cells against CFZ-induced proteotoxicity by facilitating the elimination of aggregated proteins through the autophagy-lysosome pathway [252].

Interestingly, KL4 upregulation in KMS-11/Cfz and KMS-34/Cfz cells was also associated with downregulation of genes involved in promoting B-cell differentiation. These findings echo the previously described BTZ resistance mechanisms involving alterations to the B-cell differentiation program. In this regard, mechanisms involving B-cell differentiation (i.e. Xbp-1 mutations) has not yet been validated in CFZ resistance models. Thus, further investigations are necessary to evaluate the implications of KFL4-mediated changes in B-cell differentiation and its potential relationship to previously reported mechanisms of BTZ resistance mechanisms. Additionally, KLF4 upregulation was also observed by previous studies in cell lines treated with BTZ and epoxomicin [253], suggesting that induction of KLF4 may be a common cellular response to proteasome inhibition. Thus, it would be important to examine the impact of KLF4-mediated autophagy activation on the cellular sensitivity to other PI agents as well.

2.9.3 p53-Mediated Resistance

The role of the tumor-suppressor protein p53 in PI-mediated cell death is currently not well-understood due to controversial findings regarding whether p53 is required for PI-induced apoptosis. For instance, apoptosis activation following PI treatment was found to require p53 function in renal cancer and liver cancer cells [254, 255], whereas breast cancer and mantle cell lymphoma cells activated apoptosis regardless of p53 status [69, 73, 256]. Although the expression and mutational status of p53 have been implicated in resistance to various other classes of anti-cancer agents, its impact on PI sensitivity remains unclear. In regards to this, Ling et al. reported that expression of wildtype p53 and low levels of the anti-apoptotic protein survivin were associated with increased BTZ sensitivity in colorectal cancer cells, whereas expression of mutant or null p53 along with high levels of survivin were associated with decreased BTZ sensitivity [72]. However, no further mechanisms were described regarding how BTZ resistance was regulated by p53 and survivin expression. As well, no cross-validation of the role of p53 in mediating CFZ resistance has been reported.

Walerych et al. found recently that the transcriptional activities of several gain-of-function p53 mutants were important contributors to CFZ resistance in triple-negative breast cancer and other types of solid cancer cells [257]. The study identified the proteasome machinery as a primary target of the mutant p53 transcriptional program, and demonstrated a strong correlation between high expression of proteasome genes in breast cancer patients expressing gain-of-function (GOF) p53 mutations and poor disease prognosis [257]. GOF p53 mutants were found to interact with the transcription factor Nrf2 to promote proteasome gene transcription in cancer cells, leading to increased proteasome activity and decreased sensitivity to CFZ. Abolishment of the mutant p53-Nrf2 interaction in MDA-MB-231 cells and mouse xenografts prevented CFZ-induced upregulation of proteasome genes and enhanced cell-killing compared to treatment of CFZ alone. Similar effects were observed in several other solid cancer cell lines expressing mutant p53, including hepatic, ovarian, pancreatic, and colonic cancer cells. Together, these findings revealed a novel mechanism of CFZ resistance involving the GOF p53 mutant and offered the potential utilization of APR-246, which inhibits mutant p53 activity by restoring the wildtype p53 conformation, as a potential strategy to overcome CFZ resistance [257].

2.10 Cross-Resistance of CFZ and BTZ

2.10.1 Proteasome-Dependent Mechanisms

Several of the previously proposed mechanisms underlying BTZ resistance have also been found to contribute to CFZ resistance. Verbrugge et al. reported that THP1 myeloid leukemia cells with acquired resistance to BTZ were also resistant to CFZ and two other epoxyketone-based PIs, although the extent of resistance to the latter PIs were to much less compared to BTZ [241]. These findings were later confirmed by Huber et al., who constructed yeast model systems containing the previously reported *PSMB5* mutations and examined their effects on the binding and inhibition of the proteasome by various PIs. Their findings revealed that *PSMB5* mutations which interfered with BTZ binding to the β5 subunit also decreased binding of epoxyketone PIs including CFZ. However, of the epoxyketone PIs examined, the binding and inhibitory ability of CFZ toward β5 was least affected, suggesting that *PSMB5*-mediated cross-resistance to CFZ may be less impactful compared to other PIs [154].

Alterations of proteasome expression and activity levels have been shown to affect cellular sensitivity of PIs. Downregulation of IP expression has been reported to confer resistance to BTZ, CFZ, and the IP-selective inhibitor ONX-0914 in hematological cancer cell lines including MM, leukemia, and mantle cell lymphoma cells [171, 172, 174]. Induction of IP subunits by IFN-γ as well as downregulation of constitutive proteasome subunits have shown resistance-reversing effects [171, 258]. Interestingly, downregulation of the 19S proteasome regulatory subunit was also found to decrease cellular sensitivity to both BTZ and CFZ in MM cells [259]. While the exact mechanisms through which 19S downregulation mediates PI resistance remain to be elucidated, the authors of the study hypothesized that 19S knockdown may result in the accumulation of certain cytoprotective proteins selectively. Among the proteins accumulated following 19S knockdown were key regulators of the autophagy and apoptotic pathways including SQSTM1/p62 and Mcl-1, which were also implicated in either BTZ or CFZ resistance studies previously [227, 252]. Accumulation of SQSTM1/p62 suggests that 19S downregulation may be an inducer of the autophagy-lysosome pathway, whereas increased Mcl-1 levels may provide cells with anti-apoptotic advantages in general [259]. Consistent with previously reported findings, these observations further suggest that activation of cytoprotective autophagy is an important

contributor to PI resistance.

Changes in the catalytic activities of the proteasome have also been shown to contribute to PI cross-resistance. A recent study by Kraus et al. found that upregulation of β2/2i expression and activities conferred resistance to both BTZ and CFZ in MM cells [169]. Consequently, co-inhibition of β2/2i in addition to BTZ/CFZ-mediated β5/5i inhibition resulted in synergistic killing of resistant MM cells. These findings suggested that resistant cells may become less reliant on the CT-L proteasome activities for survival compared to PI-sensitive cells. In line with this, an earlier study demonstrated that inhibition of the CT-L proteasome subunits alone was insufficient in inducing apoptosis in MM cells, and that co-inhibition of one of the other two catalytic activities (i.e. C-L or T-L) was required to achieve maximal cell-killing [168]. In this regard, our findings showed that upregulated of β1 expression was associated with acquired CFZ resistance and cross-resistance to BTZ in BxPC3 pancreatic cancer cells (chapter 4 of this dissertation work). Co-inhibition of the C-L activity sensitized resistant BxPC3 cells to CFZ and BTZ to a lesser extent, which suggested that upregulation of the C-L activity of the proteasome may be a common mechanism mediating resistance to both CFZ and BTZ. Collectively, our findings and the findings of others provide evidence for upregulation of C-L and T-L proteasome activities as a cytoprotective mechanism to compensate for the cytotoxic effects of PIs targeting the CT-L catalytic activities. These findings also support the co-targeting of non-CT-L proteasome subunits as a potential strategy to achieve superior proteasome inhibition and to overcome resistance associated with CT-L-targeting PIs.

2.10.2 UPR Activation and Alterations in Cellular Metabolism

An alternative hypothesis to PI-resistance mediated by proteasome-dependent mechanisms (e.g. *PSMB5* mutation and changes to the proteasome subunit expression and activities) is that low activation status of the UPR pathway, namely the IRE1/Xbp1 arm, may render cells less sensitive to proteasome inhibition by altering the stress response pathway and the differentiation program of plasma cells [173]. In support of this theory, low activation and mutations of the transcription factor Xbp1 were previously identified in BTZ-resistant hematological cell lines and correlated with poor clinical outcome [173]. More recently,

Soriano et al. confirmed the downregulation and mutation status of Xbp1 in both BTZ- and CFZ-resistant MM cells using proteomic analysis [245]. In addition, the authors also identified Nrf2 upregulation and increased protein chaperone capabilities (i.e. upregulation of HSP70, HSP90, and HSP105) as contributors to BTZ and CFZ resistance. These findings are complimentary to previously proposed resistance mechanisms involving upregulated proteasome transcription and enhanced ability to cope with proteotoxic stress in PI-resistant cells [176, 204, 207]. Interestingly, proteomic analysis also revealed higher rates of oxidative metabolism in BTZ and CFZ resistant MM cells, along with overexpression of key enzymes responsible for the production of NADPH. Based on these observations, the authors proposed that enhanced glycolytic rates and increased production of reducing metabolites may confer resistance to BTZ and CFZ by mitigating the oxidative stress caused by proteasome inhibition [245].

2.11 Current Understanding & Questions Remaining

Many mechanisms have been proposed for BTZ resistance over the last decade including contributions from both proteasome-dependent and proteasome-independent mechanisms. The most prevalently reported mechanisms for BTZ resistance were mutations in the *PSMB5* gene and upregulation of CP catalytic subunits. Since no clinical evidence of BTZ resistance mediated by *PSMB5* mutations have been found, this mechanism is now recognized as a potential artifact of the resistance selection process *in vitro*. In comparison, alterations in CP expression and function present more promising opportunities for therapeutic development to enhance BTZ efficacy and overcome resistance. Findings from the current dissertation (chapter 4) provide proof-of-concept evidence for the co-targeting of the C-L activity of the proteasome as a strategy to potentiate BTZ and CFZ activities in PI-resistant cells. Similarly, combined inhibition of other non-CT-L activities of CP and IP have also demonstrated promising utility in the context of PI resistance. Further development of subunit-selective PIs, especially with better selectivity between CP and IP activities, will be important to better validate the targeting or co-targeting of non-CT-L activities as viable approaches to overcome PI resistance. As well, the availability of next-generation PIs will also help advance our understanding of how alterations in proteasome expression and function may determine cellular sensitivity of PIs.

BTZ resistance mediated by Xbp-1 inactivation has also been reported by a number of studies and is perhaps supported by the most clinically relevant evidence. However, since this mechanism was mainly described in the context of B-cell differentiation, it is likely only relevant to BTZ resistance in hematological cancers. It is possible that alterations in Xbp-1 function may also affect other downstream pathways regulated by the UPR, thus it would be important to further examine whether Xbp-1 downregulation and/or inactivation may also have general cytoprotective effects independent of B-cell differentiation. Such information would be especially useful for understanding the factors involved in determining PI sensitivity in hematological vs solid malignancies.

Fewer mechanisms have been proposed for CFZ resistance, many of which have not been well-validated except for P-gp upregulation. Acquired CFZ resistance mediated by increased

P-gp transport was shown by us and several others across hematological and non-hematological cell line models, but clinical evidence has been lacking until recently. The identification of *ABCB1* upregulation in a CFZ-refractory MM patient helped further validate this mechanism as a potentially relevant one for clinical CFZ resistance. However, additional clinical evidence is still needed to confirm the prevalence and impact of P-gp upregulation in CFZ-treated patients. Further investigations are also necessary to determine whether a relationship exists between baseline P-gp expression in patients and their corresponding CFZ response in the clinic. Additionally, it is important to assess the impact of P-gp expression on the cross-resistance of CFZ and other chemotherapeutics that are known substrates of P-gp. For combination therapies; P-gp activity may also affect both the efficacy and toxicities of agents used in conjunction with CFZ.

Despite the advances made in our knowledge about the mechanisms involved in PI resistance, much remains to be learned. A general limitation of the findings summarized in this chapter is that most of the studies were carried out using *in vitro* models, with few having been validated in clinically-relevant settings. Thus, future investigations in clinically-relevant models are critical to determine whether these mechanisms play a significant role in PI resistance in patients and to identify resistance-combating treatment strategies based on such clinical findings. Additionally, many of the proposed mechanisms contributing to BTZ and CFZ resistance have been identified in models of hematological cancers, but not in solid cancer models. Strategies proposed based on these models therefore may not necessarily be relevant for PI resistance in solid cancer cells. In this regard, it is important to cross-validate the findings identified in hematological cancer cells using resistance models of solid cancer. By doing so, we may gain a better understanding of resistance mechanisms common to all cancer cells as opposed to mechanisms specific to certain cellular contexts. Gaining a better handle on how PI response is determined in solid cancer cells will not only be crucial for expanding the therapeutic utility of current PI agents, but will also provide useful insights in the design and development of next-generation PIs.

Chapter 3 DEVELOPMENT OF PEPTIDE-BASED REVERSING AGENTS FOR P-GLYCOPROTEIN-MEDIATED RESISTANCE TO CARFILZOMIB

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3.1 Introduction

The proteasome is a multiprotease complex found in all eukaryotic cells and plays a key role in regulating ubiquitin-dependent turnover of numerous proteins, including those involved in cell cycle progression, apoptosis, survival and stress response [24, 260]. For this reason, many research efforts over the past decade have been dedicated to developing proteasome inhibitors as anticancer agents, resulting in the development of BTZ, a first-in-class proteasome inhibitor approved for the treatment of relapsed multiple myeloma and refractory mantle cell lymphoma. The successful development of BTZ is followed by a number of next-generation proteasome inhibitors currently in preclinical and clinical development [2, 261]. Among them, the tetrapeptide epoxyketone CFZ is the furthest in clinical development [262]. Compared to BTZ, CFZ is shown to be highly specific for the proteasome and minimally inhibits other cellular proteases. This specificity of CFZ has been attributed to its improved toxicity profiles over BTZ, a dipeptidyl boronate, which can inhibit non-proteasomal proteases, such as a serine protease HtrA2/Omi, and cause severe side effects such as peripheral neuropathy [68, 262, 263].

Further supporting the promising potential of CFZ therapy, several investigations have now demonstrated that CFZ has excellent anti-cancer activity against hematopoietic malignancies and solid cancers preclinically [6, 262]. However, it is likely that resistance will eventually emerge and cancer cells will not retain long-term CFZ sensitivity. For epoxomicin (a prototypical peptidyl epoxyketone proteasome inhibitor isolated from an actinomycete strain), it has been shown that upregulation of P-glycoprotein (P-gp/MDR1) leads to cellular extrusion of epoxomicin and confers drug resistance [239]. For CFZ, an

early report described that human multiple myeloma cells resistant to doxorubicin are less sensitive to CFZ compared to their parental controls, suggesting that multidrug resistance (MDR)-related efflux pumps may be involved in the CFZ cross-resistance [241]. Recently, a more detailed investigation was carried out using multiple cell lines stably expressing various MDR-related transporters and the results indicated that only P-gp, but none of the other MDR-related transporters, has the ability to extrude CFZ and to confer resistance [241]. However, it remains to be determined whether P-gp upregulation serves as a major mechanism for CFZ resistance in cancer cells exposed to prolonged CFZ therapy. In case of BTZ, multiple resistance mechanisms have been reported; they include the amplification and/or mutation of target proteasomal subunits [147, 148, 150, 160], alterations in protein biosynthesis [165], or changes in ER stress response pathways [264]. An early clinical trial with CFZ reported that some patients who are refractory to BTZ remain responsive to CFZ therapy, suggesting that resistance mechanisms for CFZ may be unique from those determining BTZ resistance [262]. However, this has not been thoroughly examined.

In our current study, we investigated molecular factors involved in CFZ resistance by establishing CFZ-resistant lung and colon adenocarcinomas cell lines. Our results indicate that P-gp-mediated efflux plays a major role in acquired resistance of H23 and DLD-1 cancer cells to CFZ. As a proof of concept, we then set out to develop agents that can restore the sensitivity of cells to CFZ. We found that peptide analogs as small as dipeptides derived from the peptide backbone of CFZ can effectively restore CFZ sensitivity in our cell line models. These results indicate that small and minimally toxic peptide analogs may be used to overcome the resistance of cancer cells to CFZ or other drugs that develop P-gp-mediated drug resistance.

3.2 Materials and Methods

3.2.1 Cell lines and reagents

Human cancer cell lines H23 (lung adenocarcinoma) and DLD-1 (colon adenocarcinoma) were obtained from American Type Culture Collection and maintained in the recommended culture media of RPMI-1640 supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA)) at 5% CO2 and 37 °C. CFZ and YU-101 were synthesized and purified as reported previously [6, 122] and BTZ was obtained from ChemieTek Inc. (Indianapolis, IN). Di-, tri- and tetra-peptide analogs of CFZ were prepared following the standard peptide synthesis strategy.[265] Verapamil and paclitaxel were obtained from Sigma (St. Louis, MO). P-gp (F4) and BCRP antibodies were obtained from Sigma and GAPDH antibody was obtained from Cell Signaling (Danvers, MA). Vibrant® multidrug resistance assay kit containing calcein-AM was obtained from Invitrogen (Carlsbad, CA).

3.2.2 Establishment of CFZ-resistant cancer cell lines

H23 and DLD-1 cells were maintained with stepwise-increasing concentrations of CFZ over a period of 6 months. Initial concentrations of CFZ were 10 and 15 nM for H23 and DLD-1 cells and increased up to 500 and 1,000 nM over 6 months, respectively. The cells resistant to CFZ were termed H23/CFZR and DLD-1CFZR.

3.2.3 Cell viability assay

H23/CFZR, DLD-1CFZR and parental H23 and DLD-1 cells in logarithmic phase growth were seeded in 96-well plates at 5,000 - 20,000 cells/well in three or four replicates. After 24 hours, cells were treated with CFZ, BTZ, YU-101 or paclitaxel at a series of concentrations for 72 h. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). The IC_{50} values were calculated by fitting the observed data to sigmoidal dose-response curves with variable slopes using GraphPad Prism 5.0 (La Jolla, CA).

3.2.4 Immunoblotting

Whole cell lysates were prepared in a lysis buffer (17 mM Tris, 50 mM NaCl, 0.3% Triton X-100, pH 8.0) containing protease inhibitors (Roche Applied Science, Indianapolis, IN). Cell

lysates containing equivalent amounts of total protein were resolved by SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skim milk, membranes were probed with primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody. GAPDH was used as a gel loading control. Signals were visualized using enhanced chemiluminescence detection reagents.

3.2.5 Quantitative RT-PCR

Total RNAs (1 μg) from H23/CFZR, DLD-1CFZR and parental H23 and DLD-1 cells were converted to single-stranded cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For quantitative RT-PCR analyses of MDR1 and BCRP transcripts, the following primer sequences were used; for MDR1, sense 5′-GTCCCAGGAGCCCATCCT-3′ and antisense 5′-CCCGGCTGTTGTCTCCAT-3′; for BCRP, sense 5′-TGGCTGTCATGGCTTCAGTA-3′ and antisense 5′-GCCACGTGATTCTTCCACAA-3′; for β–actin, sense 5′-GCATCCTCACCCTGAAGTAC-3′ and antisense 5′-GATAGCACAGCCTGGATAGC-3′. Quantitative RT-PCR was performed in triplicate using iCycler with the iQ SYBR-green Supermix (Bio-Rad). The conditions for quantitative RT-PCR were as follows: annealing at 65 °C with 40 cycles for MDR1 and β–actin; annealing at 55°C with 40 cycles for BCRP. The relative quantity of the transcripts were calculated by the formula $2^{-\Delta Ct}$, where ΔCt was determined by subtracting the average β–actin Ct value from the average target Ct value.

3.2.6 Synthesis of peptide analogs structurally related to CFZ

Tetra-peptides lacking an epoxyketone pharmacophore and its truncated peptides were synthesized by standard peptide coupling methods [265]. All intermediates and final products were validated by ^{1}H NMR and mass spectrometry.

3.2.7 Impact of peptide analogs on CFZ sensitivity in CFZ-resistant cells and their parental controls

In order to examine the resistance reversing effects of peptide analogs, H23/CFZR or DLD-1CFZR cells were treated with peptide analogs (25 μM) in the absence and presence of CFZ (500 nM for H23/CFZR and 1000 nM for DLD-1CFZR). After 72 hours, cell viability was measured using the CellTiter-Glo luminescent cell viability assay and expressed as % viability relative to those treated with vehicle alone. With the selected peptide analogs

(compounds 8 to 10), we examined whether these truncated peptides can restore CFZ sensitivity of H23/CFZR or DLD-1CFZR cells in a concentration-dependent manner. Additional experiments were performed using compounds 8 to 10 to examine whether they can restore sensitivity to paclitaxel (2 μM) in H23/CFZR or DLD-1CFZR cells and whether they have any potentiating impact on cell killing by CFZ (15 nM) in the parental H23 and DLD-1 cells.

3.2.8 Comparison of peptide analogs for their inhibitory effects on the P-gp-dependent extrusion of calcein

The P-gp-inhibitory activity of peptide analogs was assessed using Vybrant® Multidrug Resistance Assay Kit (Invitrogen). Briefly, DLD-1CFZR and H23/CFZR cells were plated onto 96-well plates (300,000 cells per well) in suspension. Cells were then pre-incubated with PBS, verapamil (25 μM), or compounds 8 to 10 (25 μM) for 15 min at 37 °C. Subsequently, calcein-AM was added to the cells at a final concentration of 0.25 μM and the plates were incubated for 15 min at 37 °C. Cells were washed and cellular retention of calcein was assessed by measuring fluorescence (excitation 494 nm, emission 517 nm) using a fluorescence microplate reader (SpectraMax M5, Molecular Devices). Experiments were conducted in three replicates and the relative calcein retention was calculated by normalizing fluorescence signals from cells treated with compounds to those from cells treated with vehicle alone.

3.2.9 Statistical analyses

Results are expressed as means ± S.D. The statistical significance of the differences between groups was determined using either Student's t-test (with Bonferroni adjustment for multiple testing when appropriate) or one-way ANOVA (followed by the Newman-Keuls test or the Bonferonni test). All statistical analyses were carried out using GraphPad Prism (GraphPad Software).

3.3 Results

3.3.1 Development of H23 and DLD-1 cell lines with acquired resistance to CFZ

H23/CFZR and DLD-1CFZR cells were established by maintaining H23 and DLD-1 cells with increasing concentrations of CFZ for approximately 6 months. H23/CFZR and DLD-1CFZR cells were found to proliferate without any apparent cell death at CFZ concentrations of 500 and 1,000 nM, respectively. We determined the extent of CFZ resistance by measuring the IC_{50} values of CFZ in the resistant cell lines and found that IC_{50} for H23/CFZR and DLD-1/CFZR cells were markedly increased compared to their respective parental controls. CFZ IC_{50} was increased 74-fold for H23/CFZR cells compared to H23 parental cells (1,300 nM vs 17.6 nM) and 88-fold for DLD-1/CFZR cells compared to DLD-1 parental cells (2,900 vs 32.9 nM) (Figure 3.1 and Table 3.1).

We also determined whether H23/CFZR and DLD-1CFZR cells are cross-resistant to the closely related epoxyketone-based inhibitor YU-101 (of note, CFZ is derived from YU-101, by adding the more water-soluble morpholino group at the N-terminus) [266]. Not surprisingly, H23/CFZR and DLD-1CFZR cells were found to be highly cross-resistant to YU-101 (Figure 3.1 and Table 3.1). In contrast, H23/CFZR and DLD-1CFZR cells remained sensitive to BTZ; BTZ sensitivity in both CFZ-resistant cell lines differed only marginally compared to their respective controls. On the other hand, CFZ-resistant cells displayed a high degree of cross-resistance to paclitaxel, which is a well-known P-gp substrate (>200-fold increase in the IC_{50} values compared to parental controls).

Table 3.1 Acquired PI resistance in H23 and DLD-1 cell lines

Cell line	IC_{50} (nM)			
	CFZ	YU-101	BTZ	Paclitaxel
H23	17.6	23.7	6.3	4.7
H23/CFZRR	1300	> 1000	57.1	> 1000
DLD-1	32.9	37.7	19.8	5.5
DLD-1CFZRR	2900	> 1000	102	> 1000

IC_{50} values for CFZ, YU-101, BTZ and paclitaxel in CFZ-resistant H23 and DLD-1 cells and their parental cell lines

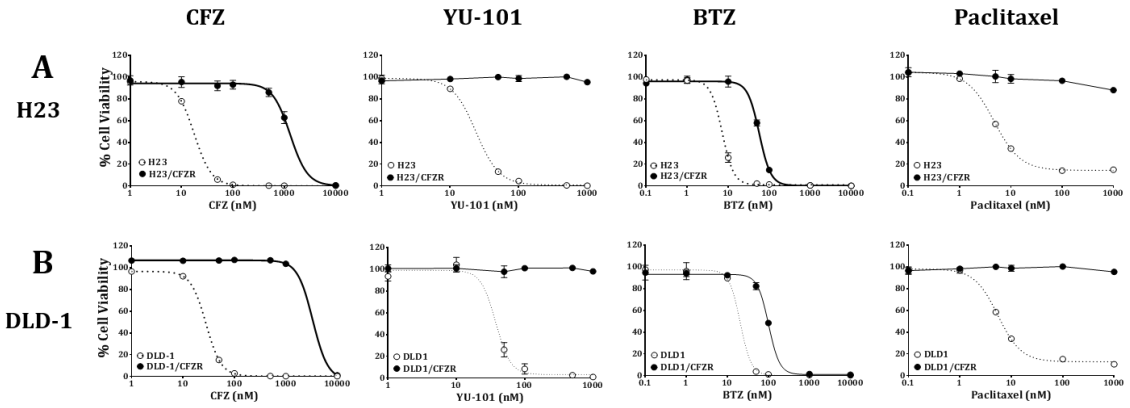


Figure 3.1 Cytotoxic effects of CFZ, YU-101, BTZ and Paclitaxel in CFZ-resistant H23 and DLD-1 cells.

H23/CFZR (A) and DLD-1CFZR (B) cells show approximately 100-fold increases in CFZ IC_{50} compared to parental controls. CFZ-resistant cells show marked cross resistance to YU-101 and Paclitaxel and slight cross-resistance to BTZ. Closed circles represent CFZ-resistant H23 and DLD-1 cells, open circles indicate their respective parental controls.

3.3.2 Upregulation of P-glycoprotein as a major mechanism for acquired resistance to CFZ

In order to verify whether decreased sensitivity of H23/CFZR and DLD-1CFZR to CFZ, YU-101, and paclitaxel is mediated by P-gp upregulation, we first compared the expression of P-gp and another multidrug resistance-related efflux protein BCRP (breast cancer resistance protein, *ABCG2*) in the CFZ-resistant and parental H23 and DLD-1 cells. Our immunoblotting and RT-PCR analyses indicated that P-gp, but not BCRP, was markedly upregulated at both the mRNA and the protein level in the H23/CFZR and DLD-1CFZR cells compared to respective controls (Figure 3.2A&B). We further examined the functional role of P-gp upregulation in determining CFZ resistance by determining the effect of verapamil, a widely used P-gp inhibitor, on cell sensitivity to CFZ. Our results showed that verapamil nearly completely abolished CFZ resistance in both H23/CFZR and DLD-1CFZR cells (Figure 3.2C&D), suggesting that P-gp is the major mediator of CFZ resistance in these cell line models.

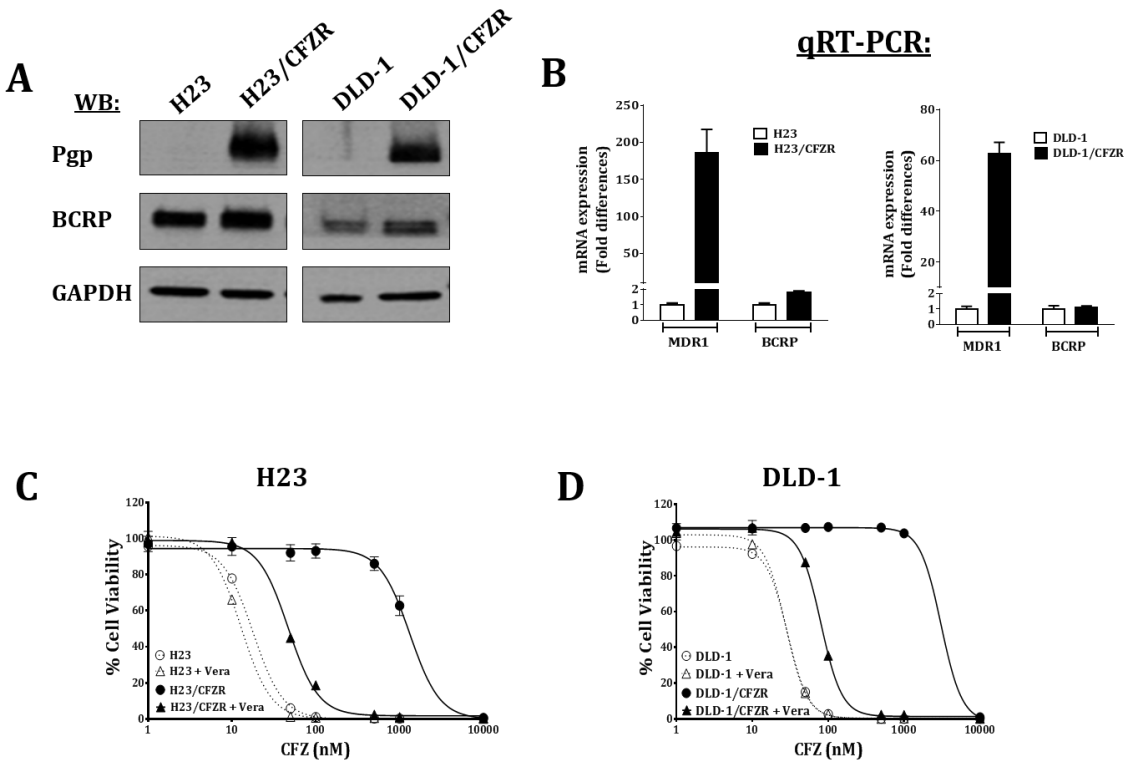


Figure 3.2 P-gp upregulation is a major mechanism of resistance in H23 and DLD-1 cells adapted to CFZ.

(A) Immunoblotting analyses showing a marked increase in of P-gp expression in H23/CFZR and DLD-1CFZR cells in comparison to their respective parental controls. (B) RT-PCR analyses showing the upregulation of *MDR1* mRNA in H23/CFZR and DLD-1CFZR cell lines in comparison to their respective parental controls. (C & D) Inhibition of P-gp using verapamil (40 μM) restores sensitivity to CFZ in H23/CFZR and DLD-1CFZR cells.

3.3.3 Small peptide analogs as resistance-reversing agents

Given that CFZ is a substrate of P-gp, we speculated that CFZ would be a good lead molecule for the development of P-gp inhibiting, resistance-reversing agents. As a first step, we synthesized a small library of peptide analogs based on the peptide backbone structure of CFZ. All of the synthesized peptide analogs had benzyl ester groups at the C-terminus instead of the epoxyketone pharmacophore (Figure 3.3A). None of these peptide analogs, when treated alone, influenced the cell viability of H23/CFZR and DLD-1CFZR (open bars in Figure 3.3B). However when co-treated with a non-lethal concentration of CFZ (500 and 1,000 nM for H23/CFZR and DLD-1CFZR, respectively), peptide analogs were able to partially restore CFZ sensitivity (Figure 3.3B). In particular, compounds 3 and 4 had the most potent resistance-reversing effect.

In order to improve their ability to reverse CFZ resistance, we next replaced the N-terminus morpholino group of these CFZ fragments with a pyridine group (compounds 5-7, Figure 3.4A). The decision for a pyridine substitution was based on recent findings which showed that peptidyl epoxyketones containing heterocylic groups at the N-terminus favor interactions with P-gp [123]. Indeed, we found that compounds 5-7 with the N-terminal pyridine substitutions have increased abilities to restore CFZ sensitivity than the previous group of compounds containing N-terminal morpholino groups (Figure 3.4B). We also confirmed that treatment of CFZ analogs did not affect cell viability, which indicated that the CFZ-sensitizing effects observed are unlikely due to the inherent toxicities of compounds 5-7 (open bars in Figure 3.4B).

Next, we aimed to increase the metabolic stability of compounds 5-7 by replaced the esterase-vulnerable benzyl ester moiety at the C-terminus with an esterase-proof Weinreb amide. The resulting group of analogs were designated as compounds 8-10 (Figure 3.5A). We found that C-terminal Weinreb amide substitutions further improved the ability of the peptide analogs to reverse CFZ resistance (Figure 3.5B). We also confirmed again that compounds 8-10 did not have major toxic effects in cells when treated alone (open bars, Figure 3.5B). Additionally, we demonstrated that the resistance-reversing effects of compounds 8 to 10 are concentration-dependent in both DLD-1CFZR and H23/CFZR cells

(Figures 3.6A and 3.6B). Compounds 8-10 were also found to restore paclitaxel sensitivity in DLD-1CFZR and H23/CFZR cells (Figure 3.6C).

To further verify whether the CFZ-sensitizing activity of these peptide analogs is related to their inhibitory effect on P-gp, we measured the impact of compounds 8-10 on the P-gp-mediated cellular extrusion of calcein. Since P-gp was found to be markedly upregulated in DLD-1CFZR and H23/CFZR cells (Figure 3.2), we expected that calcein retention would be drastically decreased assuming P-gp function was also increased in the resistant cells. Indeed, we observed that pre-incubation of the P-gp inhibitor verapamil led to an approximately 610 – 750% increase in cellular retention of calcein in both CFZ resistant cell lines (Figure 3.6D). As a control, we also examined the effect of P-gp inhibition on calcein retention in the parental DLD-1 and H23 cells. Our results indicated that verapamil had little to no effect on cellular calcein retention in parental cells (13 and 9% increases in the parental DLD-1 and H23 cells, respectively), which indicated that parental cells had much lower P-gp activity compared to resistant cells. To confirm that CFZ analogs are also transported by P-gp, we found that pre-incubation of compounds 8-10 led to substantial increases in cellular retention of calcein in DLD-1CFZR and H23/CFZR cells (Figure 3.6D). Percent calcein retention for compounds 8-10 all showed p-values of less than 0.05 when compared to the vehicle control. However, when the Bonferroni-corrected p-value threshold was used to account for multiple testing, only compounds 8 and 10 were found to be statistically significant in both DLD-1CFZR and H23/CFZR cells.

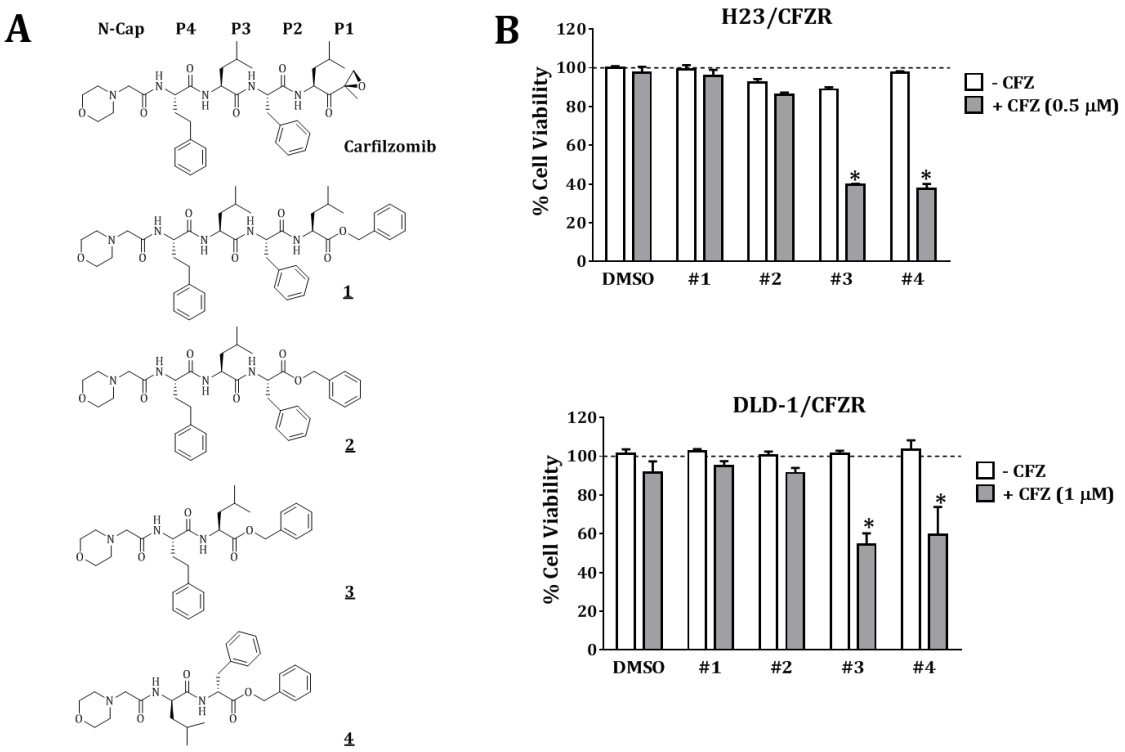


Figure 3.3 Comparison of peptide analogs with differing lengths for their reversing effects on CFZ resistance.

(A) Chemical structures of CFZ and structurally related peptide analogs, compounds 1 to 4. (B) Co-treatment of resistant cells with peptide analogs led to a partial reversal of CFZ resistance. H23/CFZR and DLD-1CFZR cells were treated with 25 μM of compounds 1-4 in the presence or absence of CFZ for 72 hours. Relative cell viability was measured using an ATP-based assay. *, $p < 0.0001$, compared to the groups treated with vehicle, CFZ, or peptide analogs alone. Statistical analysis determined by one-way ANOVA followed by Bonferroni post testing.

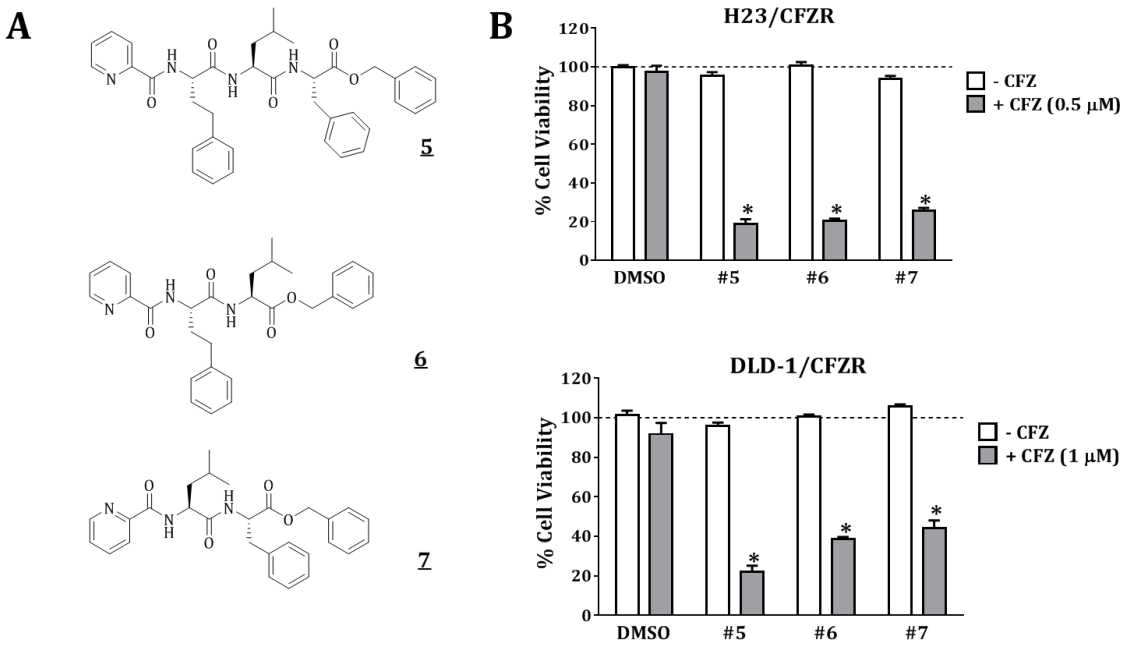


Figure 3.4 Resistance-reversing activities of CFZ analogs with pyridine substitution at the N-cap.

(A) Chemical structures of di- and tri-peptide analogs, compounds 5-7. (B) Co-incubation of di- or tri-peptide analogs with N-terminal pyridine group substitutions (compounds 5-7) restored CFZ sensitivity to a greater extent compared to their counterparts with N-terminal morpholino groups. H23/CFZR and DLD-1CFZR cells were treated with 25 μM of compounds 5 to 7 in the presence or absence of CFZ for 72 hours. Relative cell viability was measured using an ATP-based assay. *, $p < 0.0001$, compared to the groups treated with vehicle alone, CFZ or peptide analogs alone, by the one-way ANOVA, followed by Bonferroni post testing.

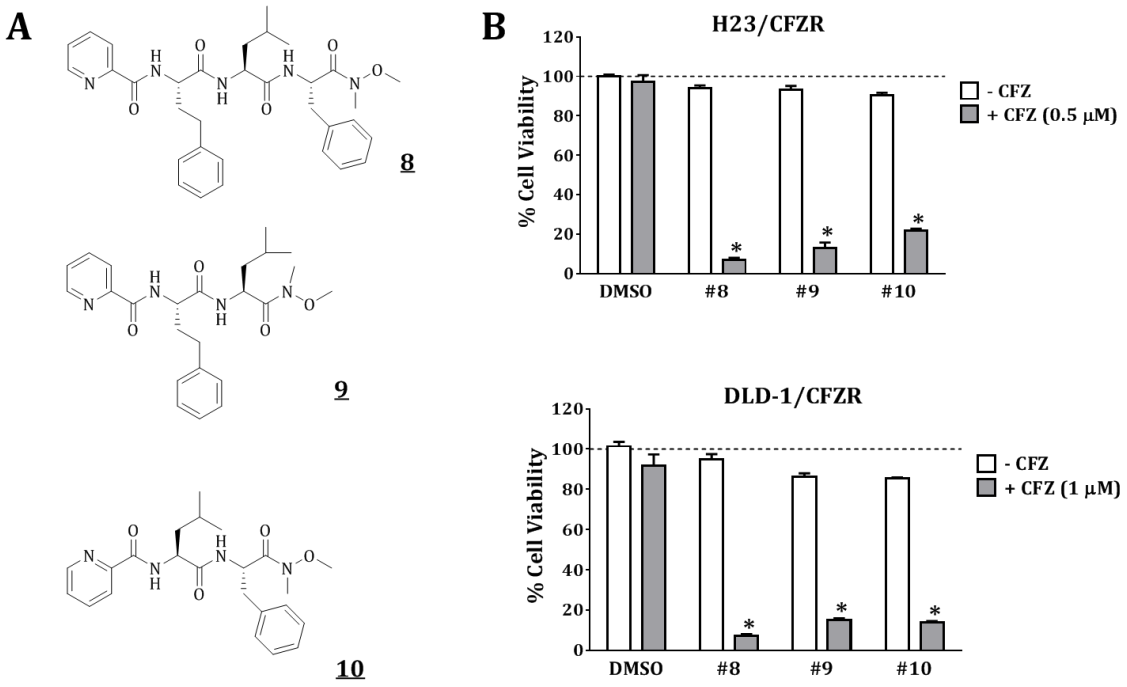
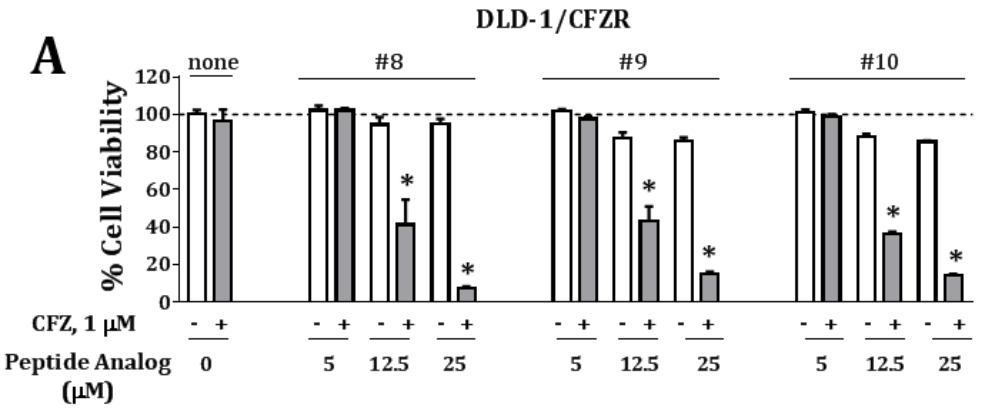
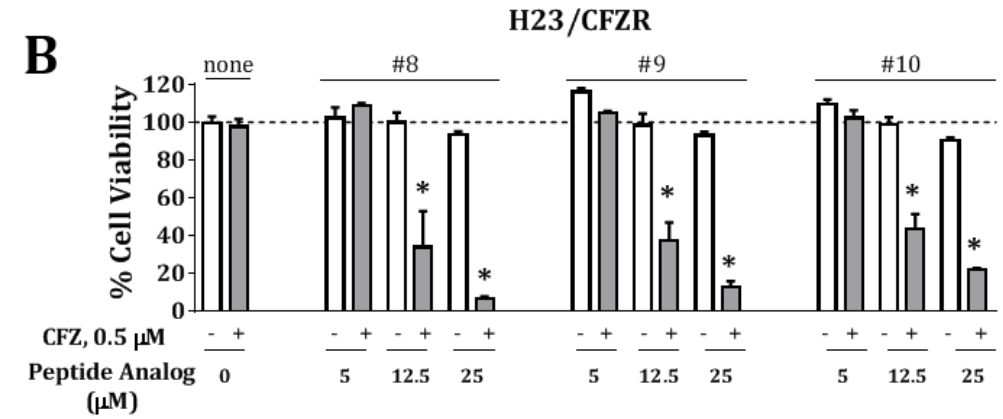
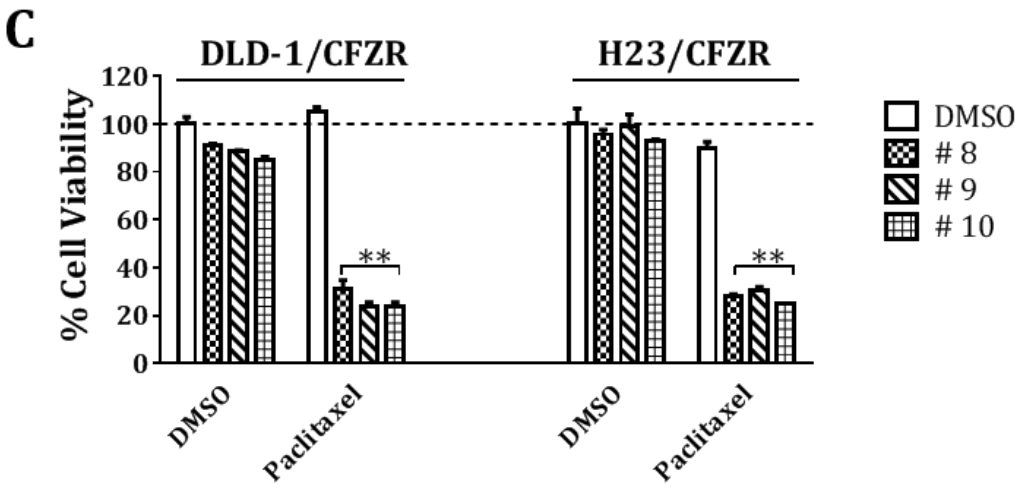


Figure 3.5 Resistance-reversing effects of peptide analogs with C-terminal Weinreb amide substitutions.

(A) Chemical structures of di- and tri-peptide analogs, compounds 8 to 10. (B) Co-incubation of di- or tri-peptide analogs with the Weinreb amide substitution (compounds 8-10) restores CFZ sensitivity to a greater extent than their counterparts with the benzyl ester group. H23/CFZR and DLD-1CFZR cells were treated with 25 μM of compounds 8-10 in the presence or absence of CFZ for 72 hours. Relative cell viability was measured using an ATP-based assay. *, $p < 0.0001$, compared to the groups treated with vehicle alone, CFZ or peptide analogs alone by the ANOVA, followed by Bonferroni post testing.







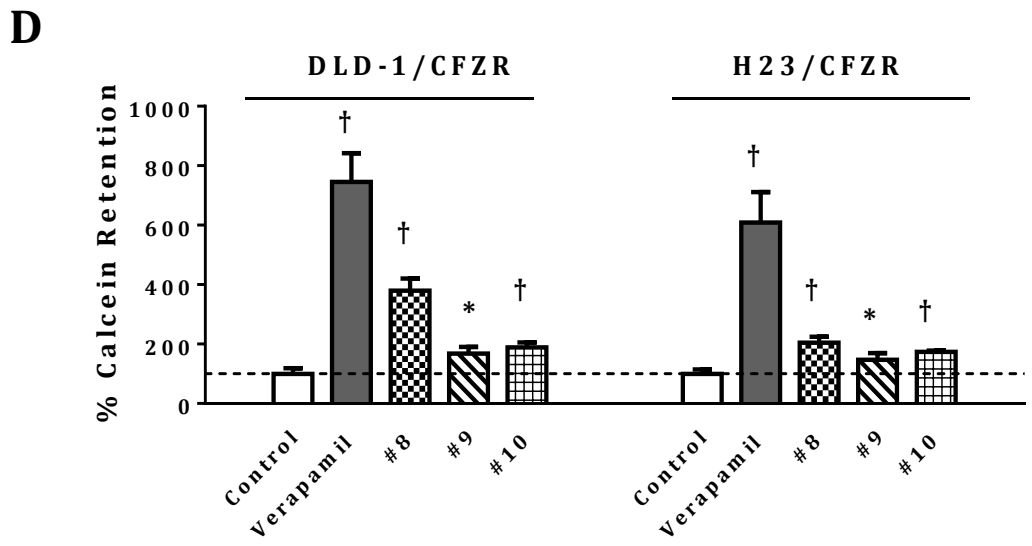


Figure 3.6 Peptide analogs 8-10 sensitize H23/CFZR and DLD-1/CFZR cells to CFZ and paclitaxel by inhibiting P-gp transport.

(A&B) Compounds 8-10 restore cellular sensitivity to CFZ in concentration-dependent manners in DLD-1CFZR (A) and H23/CFZR cells (B). *, $p < 0.001$ compared to the groups treated with vehicle alone, CFZ alone or peptide analogs alone by the one-way ANOVA followed by Bonferroni post-testing. (C) Compounds 8-10 (12.5 μM) can also reverse cross-resistance to paclitaxel (2 μM) in DLD-1CFZR and H23/CFZR cells. **, p<0.0001, compared to groups treated with vehicle alone, drug alone, or peptide analogs only by the one-way ANOVA followed by Bonferroni post testing. (D) Compounds 8-10 (25 μM) lead to increased cellular retention of P-gp substrate calcein in DLD-1CFZR and H23/CFZR cells. *, $p < 0.05$ compared to the group treated with vehicle alone by Student's t-test; † $p < 0.0125$ (Bonferroni-corrected p value threshold).

3.3.4 Impact of small peptide analogs on the CFZ sensitivity of parental cell lines

In addition to reversing the acquired resistance to CFZ, the small peptide analogs of CFZ may also potentially impact the sensitivity of parental cells to CFZ by influencing the basal P-gp expression and activity. Indeed, our results indicated that compounds 8-10 can potentiate the sensitivity of the parental DLD-1 and H23 cells to CFZ, albeit to a much lesser extent compared to CFZ-resistant cells (Figures 3.7A and 3.7B). These potentiating effects of compounds 8-10 were more pronounced in DLD-1 cells than in H23 cells, which may be related to cell line-dependent differences in the basal expression and activity of P-gp. Interestingly, higher concentrations of compound 8 (12.5 μM and 25 μM) were found to be cytotoxic in H23 cells but not in DLD-1 cells (Figure 3.7B). Given that compound 8 did not impact the cell viability of H23/CFZR and DLD-1CFZR cells (Figures 3.6A & 3.6B), it is possible that toxicity associated with H23 parental cells may be cell line-dependent.

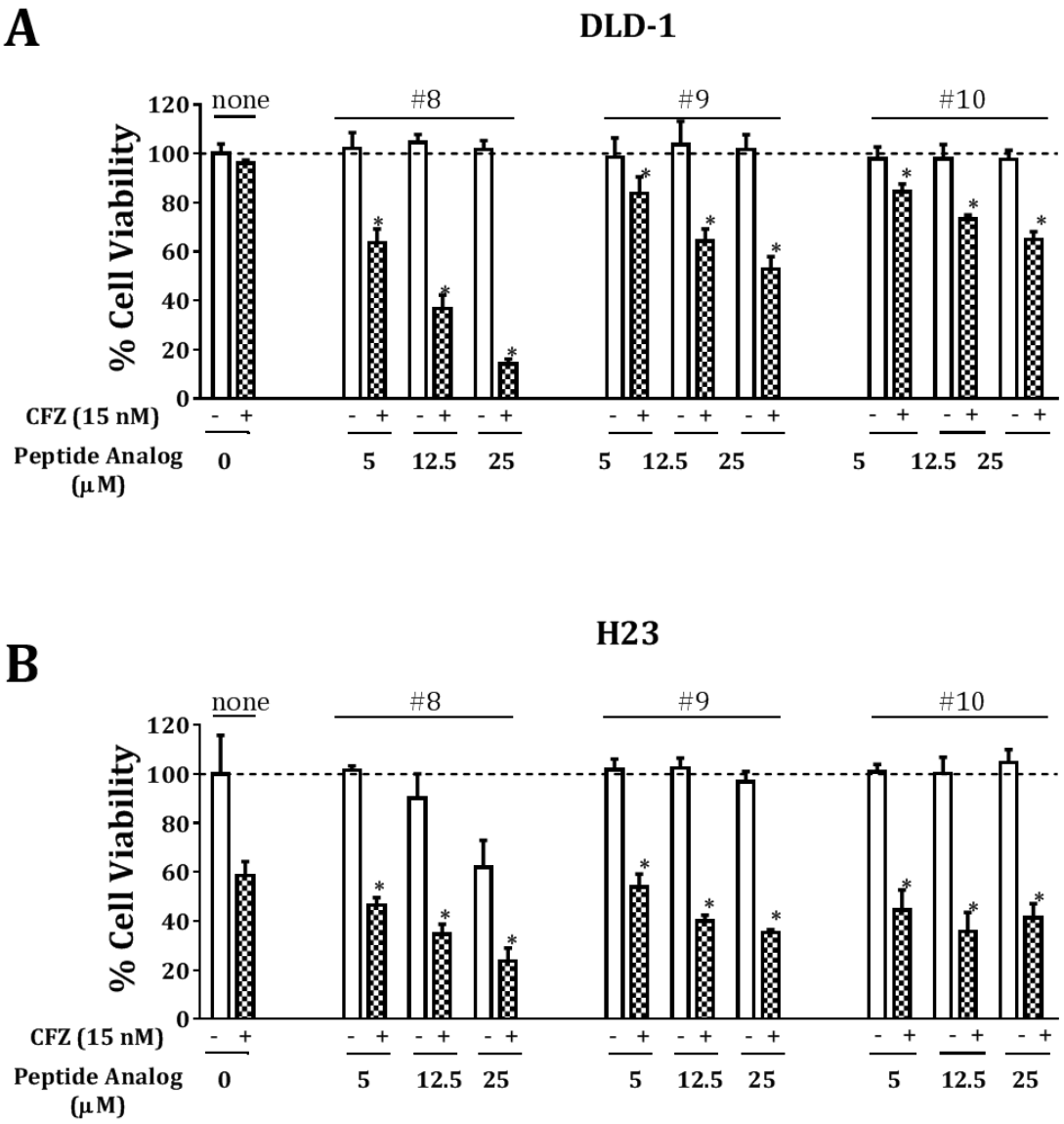


Figure 3.7 Peptide analogs 8-10 sensitize parental DLD-1 and H23 cells to CFZ.

(A & B) Compounds 8 to 10 potentiate the effect of CFZ (15 nM) in the parental DLD-1 (A) and H23 (B) cells. *, $p < 0.001$ compared to the groups treated with vehicle alone, CFZ alone or peptide analogs alone by the one-way ANOVA followed by Bonferroni post testing.

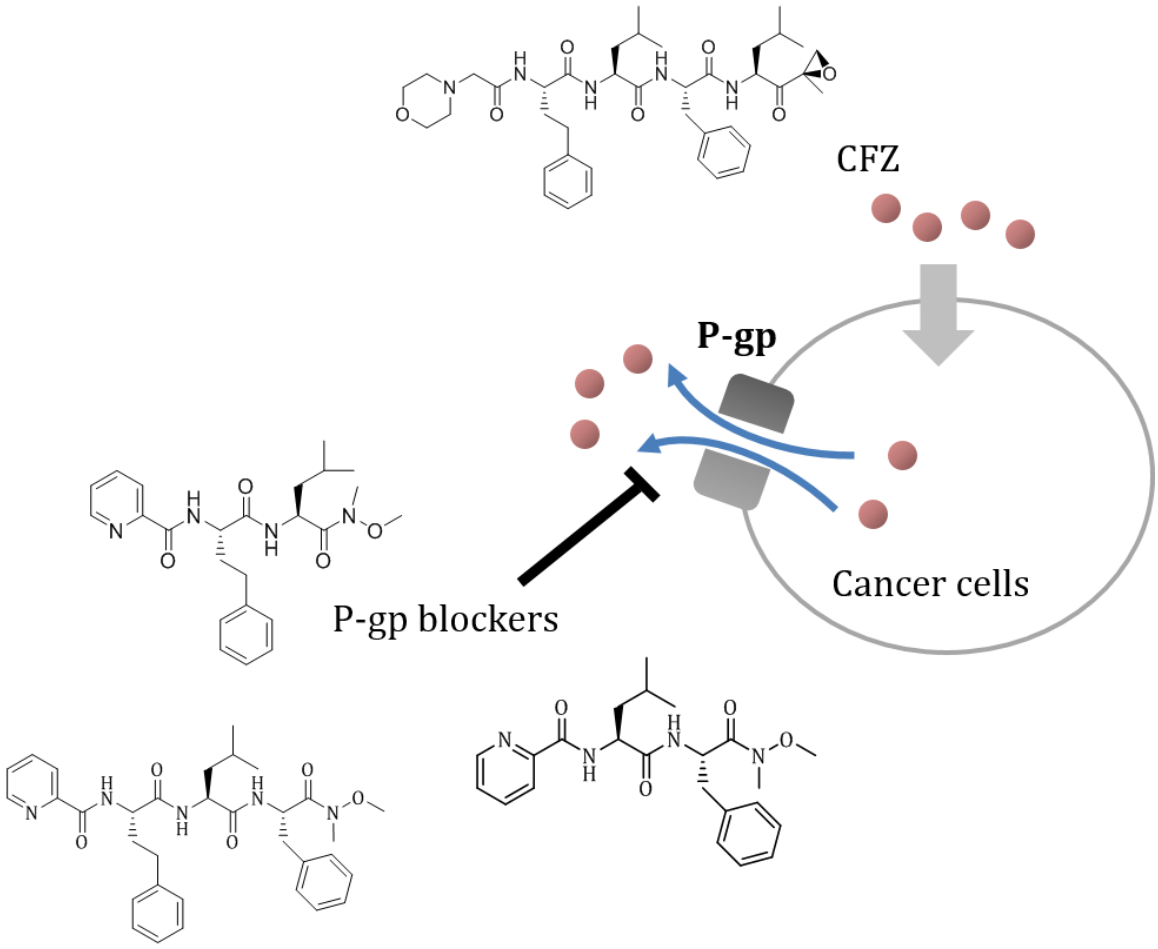


Figure 3.8 Graphic summary of P-gp-mediated CFZ resistance and resistance-reversing effects of P-gp analogs.

3.4 Discussion

CFZ is an epoxyketone-based proteasome inhibitor and has shown promising preclinical and clinical activity in MM and other types of cancer [261]. Compared to BTZ, which has also been shown to interact with non-proteasomal proteases in cells, CFZ is highly specific towards the proteasome. In early clinical trials, CFZ has shown improved toxicity profiles and superior anticancer activity over BTZ, including BTZ-refractory patients [261, 262]. These findings are encouraging and suggest that CFZ may provide additional clinical benefits as an anti-cancer treatment, especially against BTZ resistance. However, as seen with many other chemotherapy agents, CFZ therapy is also likely to fall victim to drug resistance. Since little is known about the mechanisms involved in the development of CFZ resistance, we set out to identify such mechanisms and to explore a potential strategy to restore CFZ sensitivity.

In the current study, we reported that P-gp upregulation and the resulting increased extrusion of CFZ mediate acquired resistance to CFZ in lung and colon adenocarcinoma cell line models. We also demonstrated that CFZ resistance can be reversed with co-treatment of small truncated peptides derived of CFZ (Figure 3.8). While several types of peptides, especially those containing hydrophobic side chains such as bulky aromatic and alkyl groups, have been reported to interact with P-gp [267-270], these compounds are typically much larger in size than the peptide analogs developed in our current study, and can therefore suffer solubility issues associated with having large hydrophobic moieties. Here, we report that molecules as small as dipeptide analogs can be used as resistance-reversing agents. The favorable properties of these dipeptide analogs include having relatively good water solubility and no major toxicity. While the truncated peptide analogs have shown their resistance-reversing activity in our *in vitro* models, a potential concern for their efficacy *in vivo* may be the high *in vivo* clearance typically associated with peptide fragments. In this regard, recent findings on the *in vivo* metabolism of CFZ suggest that our peptide analogs may have adequate *in vivo* stability [136]. Major metabolites of CFZ following intravenous administration of CFZ to rats were identified to be morpholino-homophenylalanine-leucine (M15) and morpholino-homophenylalanine (M14), both of

which are dipeptide fragments formed by epoxyketone hydrolysis and peptidase cleavage of CFZ. Both metabolites showed longer terminal half-lives in the plasma compared to CFZ, with M14 and M15 making up 53% of total parental dose 24 hours post-administration [136]. Based these findings, it is possible that our peptide analogs, which share close structural similarities to M14 and M15, may also have similar metabolic stability *in vivo*.

Currently, further optimization is ongoing in order to improve the efficacy and potency of the peptide analogs in reversing CFZ resistance. During the past decade, substantial advances have been made in our understanding of the structure of P-gp and its binding modes with substrates of extremely diverse structures [271, 272]. Along with that, there have been considerable efforts in the predicting and designing of P-gp substrates/inhibitors [273, 274]. Using a recently reported prediction method (a support vector machine method available from http://P-gp.althotas.com) [274], we tested whether our peptide analogs (compounds 1-10) are predicted to be P-gp substrates. The results confirmed that all ten analogs are potential P-gp substrates. However, given the possibility that the peptide analogs require micromolar concentrations to achieve resistance-reversing effects, it would be important to further optimize the analogs to improve their potency toward P-gp inhibition. Based on our experimental results, we believe that the N-terminal chemical structure of these analogs may be more important for determining P-gp interactions.

Although our present study is mainly focused on cancer cell line models with acquired resistance to CFZ, our findings can also be applied to other cases of P-gp-mediated resistance. Interestingly, we also observed that the peptide analogs can, albeit to a much lesser extent, potentiate the effects of CFZ in the parental DLD-1 and H23 cells (Figure 3.7), suggesting that cell lines with inherently high levels of P-gp may be also respond to the P-gp inhibitory effects of the peptide analogs. Thus, this may be a useful strategy to explore for intrinsic CFZ resistance that may be mediated by high baseline expression of P-gp. Alternatively, the peptide substrates may also be useful for improving the oral bioavailability of proteasome inhibitors and other therapeutic agents that are susceptible to P-gp transport. In this regard, Zhou et al. recently reported the design and synthesis of a novel, orally bioavailable epoxyketone-based proteasome inhibitor by making chemical modifications to its side chain moieties in order to evade P-gp interactions [123]. The

resulting compound, PR-047, has a much improved orally bioavailability profile (approximately 39% in rodents and dogs) compared to CFZ, likely due to reduced drug efflux by P-gp in the intestines CFZ [123].

In line with the early clinical evidence showing that CFZ can overcome BTZ resistance in the clinic [261, 262], our results support that cellular resistance to CFZ and BTZ may occur via independent mechanisms. Compared to the drastic shift in CFZ sensitivity, BTZ cross-resistance was only observed modest levels in DLD-1/CFZR and H23/CFZR (Figure 3.1). While we are not aware of any report directly examining BTZ as a potential P-gp substrate, there are several reports indicating BTZ as a poor P-gp substrate if at all. For example, whereas P-gp-expressing leukemic cells (CEM/VLB) were found to be markedly resistant to CFZ (114-fold increase in IC_{50}) compared to control, BTZ IC50 only increased by 4.5-fold [241]. Furthermore, P-gp inhibition by the peptide inhibitor P121 drastically reversed CFZ resistance in the same cell lines, whereas the effect on BTZ sensitivity was marginal [241]. Similar evidence was also reported by several other groups [147, 149, 275]. Thus, it is unlikely that P-gp upregulation is a contributing mechanism to BTZ resistance.

3.5 Conclusion

We report that cancer cells can develop acquired resistance to CFZ by upregulating P-gp expression and activity and that peptide analogs of CFZ can be used to restore sensitivity. These findings provide a potential strategy to overcome CFZ resistance or enhance CFZ activity in cells. The peptide analogs described here may provide useful structural scaffolds for the design of novel MDR reversing agents. Furthermore, findings from this study provide important insight into the potential role of P-gp in determining CFZ sensitivity. Such information should be taken into account for the development of next-generation PI agents.

Chapter 4 INHIBITION OF THE CASPASE-LIKE PROTEASOME ACTIVITY RESTORES SENSITIVITY IN CARFILZOMIB-RESISTANT CANCER CELL LINES

4.1 Introduction

The proteasome is the central player of the ubiquitin-proteasome system (UPS), a highly regulated protein degradation pathway that regulates a myriad of cellular processes, including those crucial to cancer pathogenesis [1, 276]. With the blockbuster success of the first-generation proteasome inhibitor (PI) agent BTZ (BTZ, Velcade®) [277] and its revolutionary impact on the multiple myeloma (MM) treatment paradigm [4], the proteasome was validated as a bona fide anti-cancer target. However, despite the clinical success of BTZ, limitations associated with its use such as neurotoxic effects and resistance hampered its clinical utility. To address some of these drawbacks, a second-generation PI agent, CFZ (CFZ, Kyprolis ®), was approved in 2012 and has since become indispensable in the MM armamentarium [9]. Unlike BTZ, a dipeptide boronic acid that binds to the proteasome reversibly [25], CFZ is a tetrapeptide epoxyketone which covalently binds to the catalytic threonine residues of proteasome β-subunits with improved selectivity [6]. Likely due to these differences, CFZ displays improved safety and efficacy profiles over BTZ in both BTZ-naïve and BTZ-refractory MM patients [113].

As with most other chemotherapies, drug resistance is a critical challenge in CFZ therapy. Both *de novo* resistance in CFZ-naïve patients and acquired resistance in patients receiving prolonged CFZ therapy have been observed in the clinic [11, 15]. Furthermore, reasons underlying the lack of CFZ activity in patients with solid cancers remain elusive [126, 278]. In order to better address the question of why certain patients do not respond to CFZ therapy, it is crucial to understand the mechanisms involved in determining CFZ response. Several mechanisms of BTZ resistance have been proposed, including mutations in the target proteasome subunit β5 [146-148] as well as alterations to the ER stress-sensing [208] and the unfolded protein response pathways [173, 193]. However, it is unknown whether these resistance mechanisms would be applicable to CFZ. In cell line models of CFZ resistance, upregulation of the efflux transporter P-glycoprotein (P-gp) and changes in the

lyososomal-autophagy protein degradation pathway have been reported, but these have yet to be validated for their clinical relevance [240-242, 252]. Overall, much more remains to be investigated regarding factors determining clinical CFZ response.

Two major types of proteasomes exist in mammalian cells: the constitutive proteasome (CP) and the immunoproteasome (IP). CP is normally expressed in all cell types and contains three catalytic subunits β1, β2, and β5, which display differential substrate preferences, often referred to as caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (CT-L) activities, respectively [279]. IP is predominantly expressed in cells of hematopoietic origin and consists of a distinct set of three catalytic subunits β1i, β2i, and β5i in place of their constitutive counterparts. Both types of proteasomes have been reported to be upregulated in cancer cells, likely due to their rapid proliferation and high demand of proteasome activity [57, 280]. As well, alterations in proteasome catalytic activity profiles have been noted in cancer cells exposed to or adapted to PI [149, 170]. However, it is unclear whether these changes are part of a cellular response to proteasome inhibition or serve as driving factors of PI resistance.

In the current study, we set out to investigate the mechanisms of CFZ resistance using two CFZ-adapted cancer cell lines, BxPC3 (human pancreatic cancer) and RPMI8266 (human MM). We observed that CFZ-resistant BxPC3 and RPMI-8226 cells displayed a substantial increase in the expression and activity of the β1/1i (C-L) catalytic subunits compared to parental controls. When CFZ-resistant BxPC3 cells were challenged with CFZ, we found that all proteasome activities were inhibited to near completion with the exception of the C-L activity. We utilized both chemical inhibition and genetic knockdown approaches to assess the effect of C-L proteasomal inhibition on CFZ sensitivity. Our results indicated that blockade of C-L proteasome activity potentiated the anti-cancer activity of CFZ in both BxPC3/CFZR and RPMI-8226/CFZR cells. Taken together, our findings are the first to demonstrate a potential role for the proteasomal C-L activity in acquired CFZ resistance and the selective C-L activity inhibition as an effective strategy to enhance CFZ efficacy and restore CFZ sensitivity. Such information may be important for the development of next-generation PIs as well as potential strategies to overcome CFZ resistance in the clinic.

4.2 Materials and Methods

4.2.1 Cell Lines and Reagents

Human cancer cell lines BxPC3 (pancreas adenocarcinoma) and RPMI-8226 (B-lymphocyte) were obtained from American Type Culture Collection (ATCC) and grown under recommended culture conditions at 37°C and 5% CO_2. BxPC3 cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA) and RPMI-8226 cells were maintained in RPMI-1640 media supplemented with 15% fetal bovine serum, respectively. CFZ and BTZ were purchased from LC Laboratories (Woburn, MA). Human recombinant IFN-γ was obtained from eBioscience (San Diego, CA). Chemical probes targeting β1/1i proteasome subunits Ac-PAL-ek (PAL) and YU102, and fluorogenic proteasome substrates Ac-PAL-AMC, Ac-ANW-AMC, Ac-nLP-nLD-AMC, Ac-WLA-AMC were synthesized in-house following standard peptide synthesis schemes. Ac-RLR-AMC was purchased from Bostom Biochem, Inc. (Cambridge, MA). Human recombinant IFN-γ was obtained from eBioscience (San Diego, CA). Antibodies targeting specific catalytic subunits were purchased as following: β1 from Thermo Fisher Scientific (Waltham, MA); β1i and β5 from Santa Cruz Biotechnology (Dallas, TX); β2 from Enzo Life Sciences (Farmingdale, NY); β5i from Abcam (Cambridge, UK). β-actin antibody was obtained from Cell Signaling (Danvers, MA). Cell Titer-Glo and Cell Titer Aqueous One reagents were purchased from Promega (Madison, WI). ON-TARGETplus SMARTpool PSMB6 and scramble siRNA were obtained from Darmacon (Lafayette, CO). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA).

4.2.2 Cell Viability Assay

CFZ-resistant sublines (BxPC3/CFZR and RPMI-8226/CFZR) and their parental controls (BxPC3/P and RPMI-8226/P) were seeded into 96-well plates at a starting density of 10,000 cells/well. Following overnight incubation, cells were exposed to serially diluted drug solutions. Cell viability was measured 72 h after drug treatment using either Cell TiterGlo or the Cell Titer Aqueous One cell viability assay reagents according to manufacturers' protocols. Luminescence was measured using a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA) and absorbance at 490 nm was

measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Results were analyzed using GraphPad Prism (La Jolla, CA).

4.2.3 Immunoblotting

Cell lysates were prepared using a previously reported proteasome extraction lysis buffer (50 mM Tris-HCl, pH7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA, and 0.025% digitonin) [281]. Protein concentrations of lysates were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Lysates containing equivalent amounts of total protein were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked using 5% milk or BSA according to manufacturers' protocols. Targeted proteins were detected using respective primary antibodies and appropriate secondary antibodies conjugated with horseradish peroxidase. Immunoreactive signal was visualized using Pierce ECL western blotting substrate (Thermo Fisher Scientific, Waltham, MA).

4.2.4 Proteasome Activity Assay

Using subunit-selective fluorogenic peptide substrates, activities of individual proteasome catalytic subunits were measured by monitoring the rate of substrate hydrolysis indicated by increasing fluorescence intensity over time [281, 282]. Briefly, cell lysates were prepared in digitonin-based lysis buffer (DLB; 20 mM Tris/Cl, 0.5 mM EDTA, pH 8.0) adapted from the previously published proteasome lysis buffer [281]. Protein lysates (5 μg of total protein/well) were added to 96-well microplates and enzymatic reactions were initiated with the addition of proteasome substrates, data recording immediately followed. Fluorescence signals were recorded over 60 minutes at one reading per one minute using a SpectraMax M5 microplate reader at excitation/emission wavelengths of 360/460 nm. Proteasome substrate concentrations used were as following: Ac-PAL-AMC (β1i activity, 100 μM), Ac-ANW-AMC (β5i, 100 μM), Ac-RLR-AMC (β2/2i, 20 μM), Ac-nLPnLD-AMC (β1, 100 μM), and Ac-WLA-AMC (β5, 20 μM).

4.2.5 siRNA-Mediated PSMB6 Knockdown

Cells were transfected with PSMB6-targeting siRNA or scrambled siRNA as control according to manufacturer instructions. Briefly, BxPC3/CFZR cells were plated at a starting

density of $1x10^6$ cells in a Corning 60mm TC-treated culture dish (Corning, NY) and allowed to attach for 24 h. Cells were treated with 125 nmole of siRNA prepared with Lipofectamine2000 following the recommended transfection protocol. Fresh media was provided every 24 h and cells were subcultured to maintain growth to approximately 70% confluency. Samples were collected 48 h following transfection and lysed using DLB.

4.3 Results

4.3.1 Establishment of BxPC3/CFZR Cell Line with Acquired CFZ Resistance

BxPC3/CFZR cells were established by exposing BxPC3/P cells to increasing concentrations of CFZ. BxPC3 cells adapted to grow in the presence of 100 nM CFZ appeared to have growth rates and cell morphology similar to parental controls (BxPC3/P). BxPC3/CFZR cells displayed resistance to both CFZ and BTZ (Figure 4.1A & 4.1B), as indicated by an approximately 2.3-fold increase in CFZ IC_{50} and 4.6-fold increase in BTZ IC_{50} compared to BxPC3/P cells (Table 4.1). Previously reported mechanisms involving mutations in the β5-encoding *PSMB5* gene [147, 148] and upregulation in P-gp activity [241, 242] were ruled out as potential contributors to the PI resistance observed in the current BxPC3/CFZR model.

4.3.2 BxPC3/CFZR Cells Exhibit Altered Proteasome Catalytic Subunit Expression and Activity

We examined whether there were any differences in the proteasome activities between BxPC3/CFZR and BxPC3/P cells. We first compared the levels of baseline activities of individual proteasome subunits in BxPC3/CFZR cells to those of BxPC3/P cells. We measured the proteasome activities of these drug-withdrawn BxPC3/CFZR cells (grown without the drug for up to one week) and compared them to those of parental control cells. Substantial differences in the proteasome activity profile of BxPC3/CFZR cells were observed compared to BxPC3/P controls. Notably, β1 and β5 activities in BxPC3/CFZR cells were substantially enhanced compared to parental counterparts while the activities of β1i and β5i were markedly decreased (Figure 4.1C). On the other hand, the T-L activity attributed to β2 and β2i remained unchanged. It should be noted that the T-L activity measured here is indicative of the cumulative β2/β2i activity, as the currently available probe substrate cannot differentiate between the active sites of the β2 and β2i subunits [282, 283]. Next, we investigated whether alterations in proteasome activities were due to changes in catalytic subunit expression. Our immunoblotting data showed elevated β1 and β5 levels and decreased β1i and β5i levels in BxPC3/CFZR cells compared to parental cells (Figure 4.1D), consistent with changes observed in proteasome activities (Figure 4.1C). We observed a marked increase in β2 expression but were unable to detect β2i expression due

to the lack of commercially available β2i-specific antibodies. Given that the overall T-L activity (attributed to both β2/β2i subunits) was unchanged in BxPC3/CFZR cells, the expression and activity of β2i is likely downregulated.

Table 4.1 CFZ and BTZ sensitivities in BxPC3/P and BxPC3/CFZR cells

Cell Line	IC_{50} (nM)	
	Carfilzomib	**Bortezomib**
BxPC3/P	54.5	10.4
BxPC3/CFZR	124 (2.3x)	48 (4.6x)

Comparison of CFZ and BTZ sensitivities in BxPC3/CFZR cells relative to parental controls. IC_{50} values are summarized along with fold changes (numbers in parenthesis).

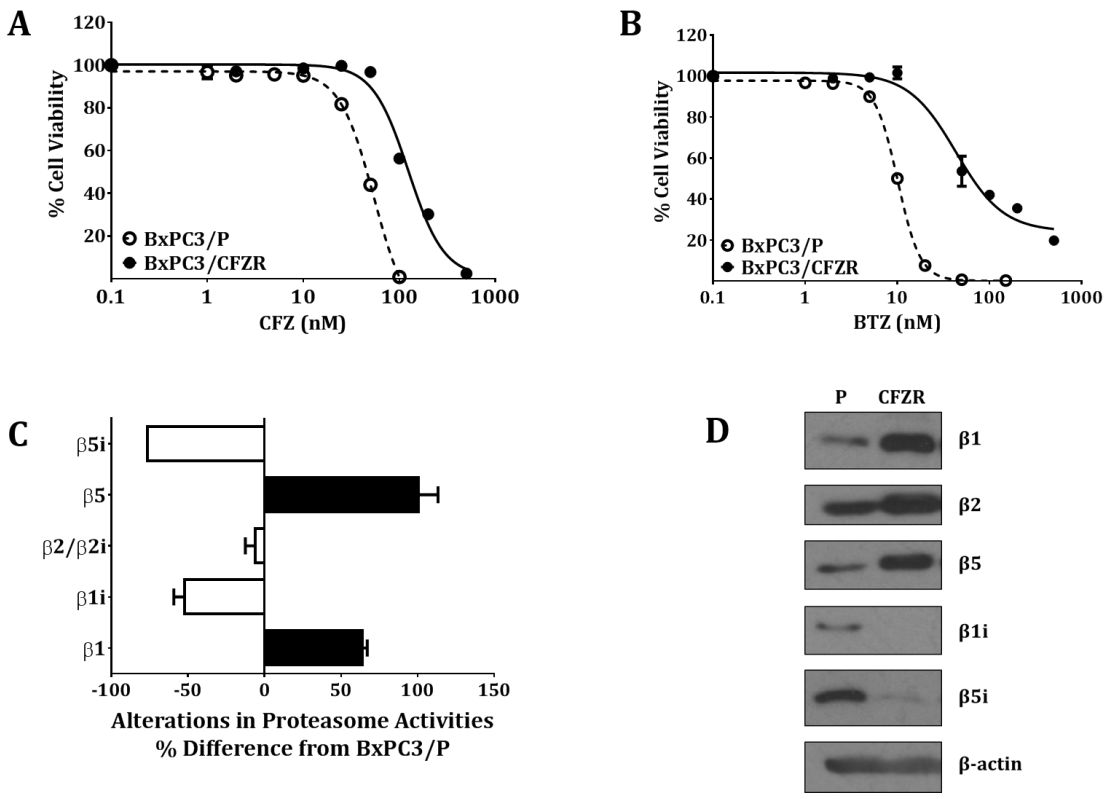


Figure 4.1 Proteasome activity and expression profiles in BxPC3/CFZR cells.

BxPC3 human pancreatic cancer cells were grown in the presence of carfilzomib (CFZ) and the adapted subline BxPC3/CFZR are resistant to both CFZ and bortezomib (BTZ). Relative cell viability data of BxPC3/P (open circles) and BxPC3/CFZR cells (solid circles) in response to CFZ (A) and BTZ (B). Cell viability results were normalized to no treatment control and shown as mean ± SEM. (C) BxPC3/CFZR cells displayed increased β1 and β5 activities and decreased β1i and β5i activities compared to BxPC3/P control. Proteasome activities were measured using subunit-selective fluorogenic substrates in cell lysates collected from resistant or parental cells maintained in the absence of CFZ for 1 week. Percent values are represented as mean ± SEM. (D) BxPC3/CFZR cells exhibited higher β1, β2, and β5 expression levels compared to BxPC3/P cells. BxPC3/CFZR lysates were collected from cells maintained in the absence of CFZ for 1 week.

4.3.3 Induction of IP Expression Sensitizes Cells to CFZ

Based on the marked decreases in β1i and β5i levels of expressions and activities in BxPC3/CFZR cells, we wanted to examine whether the restoration of β1i and β5i levels in BxPC3/CFZR cells may affect CFZ sensitivity. We exposed BxPC3/CFZR cells to IFN-γ, a known inducer of the IP catalytic subunits, and found an expected increase in β1i and β5i IP expression (Figure 4.2A), as well as in β5i activity (Figure 4.2B). Consistent with the previously reported effects of IFN-γ, we also detected a concurrent decrease in constitutive proteasome subunits compared to untreated cells (Figure 4.2A). Pretreatment with IFN-γ sensitized BxPC3/CFZR cells to both CFZ- and BTZ-induced cytotoxicity (Figure 4.2C). Similar sensitizing trends were also observed in BxPC3/P cells, however our results also showed that IFN-γ treatment alone had some inherent toxicity toward BxPC3/P cells (Figure 4.2D). Together, these findings suggest that proteasome composition of cells may be an important factor determining PI sensitivity in both parental and resistant cells. Additionally, our results suggest that high constitutive proteasome expression may be favorable for BxPC3 survival against CFZ- and BTZ-induced cytotoxicity, whereas high IP expression may be associated with increased PI sensitivity.

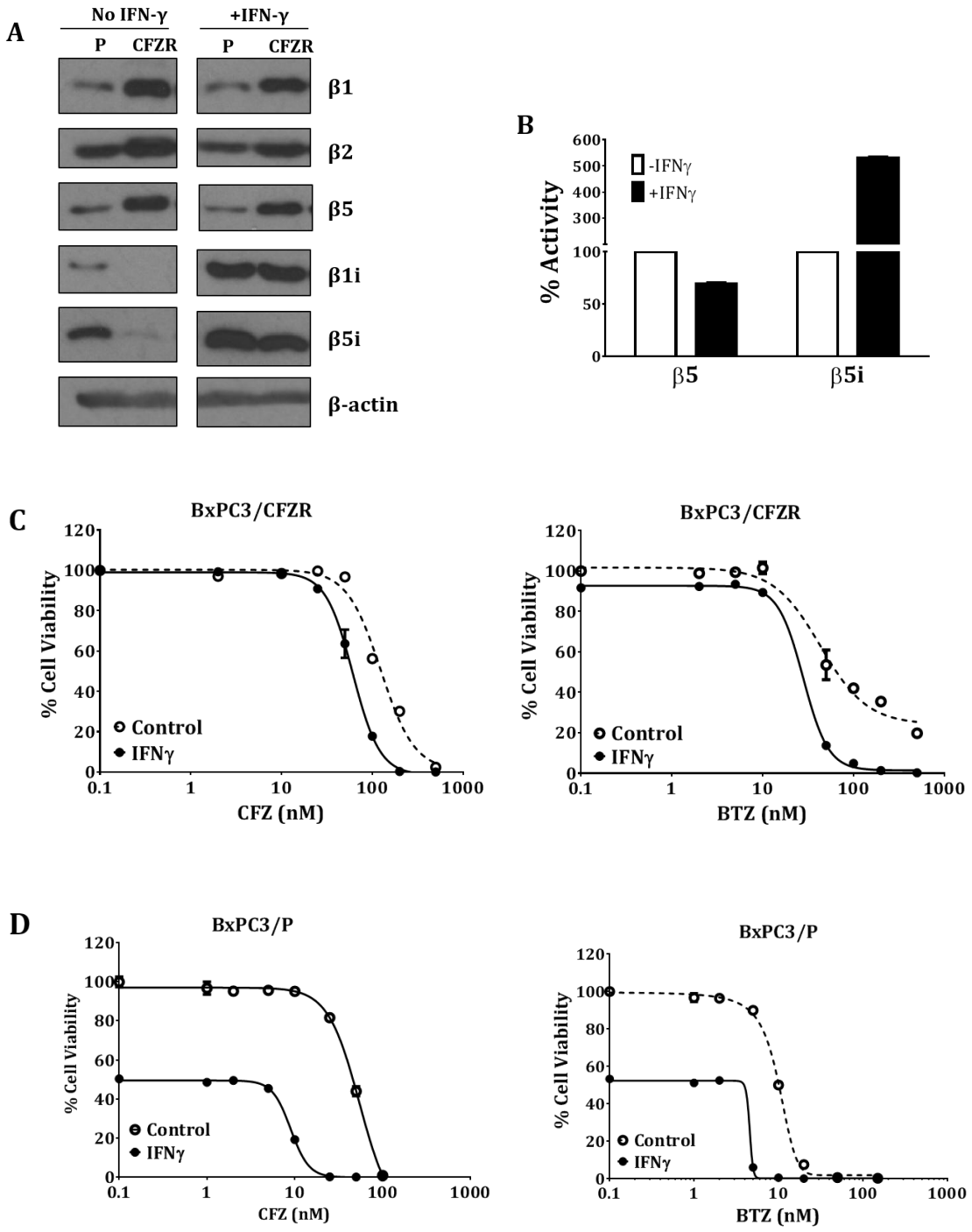


Figure 4.2 IFN-γ-induced alterations in proteasome composition impact CFZ sensitivity. (A) Treatment with 150 U/ml IFN-γ induced expression of IP catalytic subunits and downregulated CP catalytic subunits in both BxPC3/P and BxPC3/CFZR cells. (B) β5i

activity increased approximately 6-fold upon IFN-γ treatment, whereas β5 activity was decreased by approximately 1.5-fold. (C) IFN-γ treatment sensitized BxPC3/CFZR cells to both CFZ and BTZ. (D) Pretreatment of IFN-γ sensitized BxPC3/P cells to CFZ and BTZ. IFN-γ treatment alone was also toxic to BxPC3/P cells. Data from 72h viability are normalized to no treatment control, percent values are shown as mean ± SEM.

4.3.4 C-L Activity of the Proteasome is Conserved in BxPC3/CFZR Cells Treated with CFZ

In addition to assessing the basal proteasome activity profiles of BxPC3/CFZR cells grown in the absence of CFZ, we also compared the proteasome inhibitory profiles of BxPC3/CFZR and parental cells in response to CFZ. BxPC3/P or BxPC3/CFZR cells grown in the absence of CFZ for one week were treated with 100 nM CFZ and individual proteasome activities were assessed 24 h following treatment. Since 100 nM CFZ does not significantly affect BxPC3/CFZR growth, we were also able to continue following the changes in proteasome activities at 48 and 72 h after CFZ treatment. All proteasome catalytic activities were substantially inhibited in BxPC3/P cells 24 h following CFZ treatment. Similarly, all proteasome activities were inhibited considerably, with the exception of β1 activity (Figure 4.3). Remarkably, β1 activity in BxPC3/CFZR cells was both upregulated at the baseline level (163.9% of BxPC3/P) and preserved in the presence of CFZ (115% of BxPC3/P after 24 h exposure to CFZ) (Table 4.2). We observed further upregulation in β1 activity at 72 h following CFZ treatment, as well as recuperation of β5 activity (Table 4.2). These observations prompted us to examine whether the preservation of β1 activity may be a contributing factor to BxPC3/CFZR cell survival against cytotoxic proteasome inhibition by CFZ.

Table 4.2 Comparison of proteasome inhibitory profiles following CFZ treatment in parental and CFZ-resistant BxPC3 cells

Time following CFZ treatment	% Proteasome Activity of BxPC3/P Baseline				
	β1	**β2/β2i**	**β5**	**β1i**	**β5i**
0 h	163.9	93.9	200.7	47.7	23.4
24 h	115.0	54.6	41.7	24.0	13.4
72 h	157.7	85.8	177.9	70.0	35.5

Summary of the proteolytic activities of individual proteasome catalytic subunits in BxPC3/CFZR cells following 0, 24, and 72 h of CFZ treatment (100 nM). Percent proteasome activities are normalized to each respective BxPC3/P baseline activity in order to reflect both changes to the baseline activities and the activity inhibitory profiles of BxPC3/CFZR cells. Values are represented mean of three replicates.

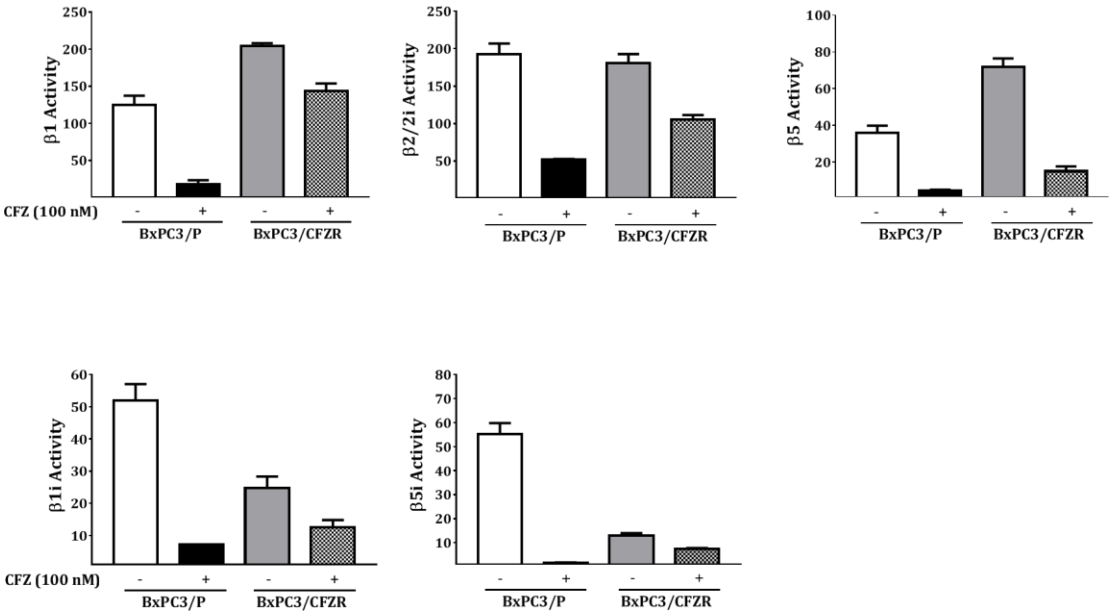


Figure 4.3 CFZ-mediated inhibition of proteasome catalytic activities in BxPC3/P vs BxPC3/CFZR cells.

β1 activity is preserved in BxPC3/CFZR cells after exposure to 100 nM CFZ. BxPC3/P or BxPC3/CFZR cells cultured in the absence of CFZ for 1 week were treated with 100 nM CFZ. All proteasome activities were substantially inhibited in both BxPC3/P and BxPC3/CFZR cells in response to CFZ, with the exception of β1 activity in BxPC3/CFZR cells. Cell lysates were collected 0 or 24 h following CFZ treatment and proteasome activities were measured using subunit-selective fluorogenic proteasome substrates. Results are presented as rates of substrate hydrolysis indicating proteasome activity, values represented graphically as mean ± SEM.

4.3.5 Chemical Inhibition of C-L Activity Sensitizes BxPC3/CFZR Cells to CFZ

In order to investigate the effect of inhibiting the C-L activity of the proteasome on the CFZ sensitivity of BxPC3/CFZR cells, we used two proteasome inhibitors YU102 and Ac-PAL-ek (PAL) that target the β1/ β1i subunits responsible for the C-L activity (Figure 4.4) [266, 284]. To first determine the potency and selectivity of YU102 and PAL in BxPC3/CFZR cells, we assessed the impact of YU102 and PAL treatment on the different catalytic activities of the proteasome. Treatment with either YU102 (3 μM) or PAL (500 nM) in BxPC3/CFZR cells lead to nearly complete blockade of the β1/ β1i activities without significantly affecting activities of the other catalytic subunits (Figure 4.4). At 10 μM, YU102 modestly inhibited β5/5i activities (Figure 4.4A), whereas PAL resulted in an enhancement of the β5 activity (Figure 4.4B).

In subsequent experiments, we used YU102 and PAL at the β1/ β1i-selective concentrations determined above to further assess the effect of inhibiting the C-L activity on the CFZ sensitivity of BxPC3/CFZR cells. Treatment of 3 μM YU102 or 500 nM PAL did not have any cytotoxic effects in BxPC3/CFZR cells (Figure 4.4C, blank bars). This is consistent with previous reports suggesting that inhibition of C-L activity alone does not impact cell viability [168]. Conversely, co-treatment of BxPC3/CFZR cells with a non-toxic concentration of CFZ (50 nM) and YU102 or PAL resulted in enhanced cytotoxic effects (Figure 4.4C, filled bars) compared to either agent alone. Additionally, YU102 also sensitized BxPC3/CFZR cells to BTZ (Figure 4.4D), albeit to a lesser extent than effects observed with CFZ.

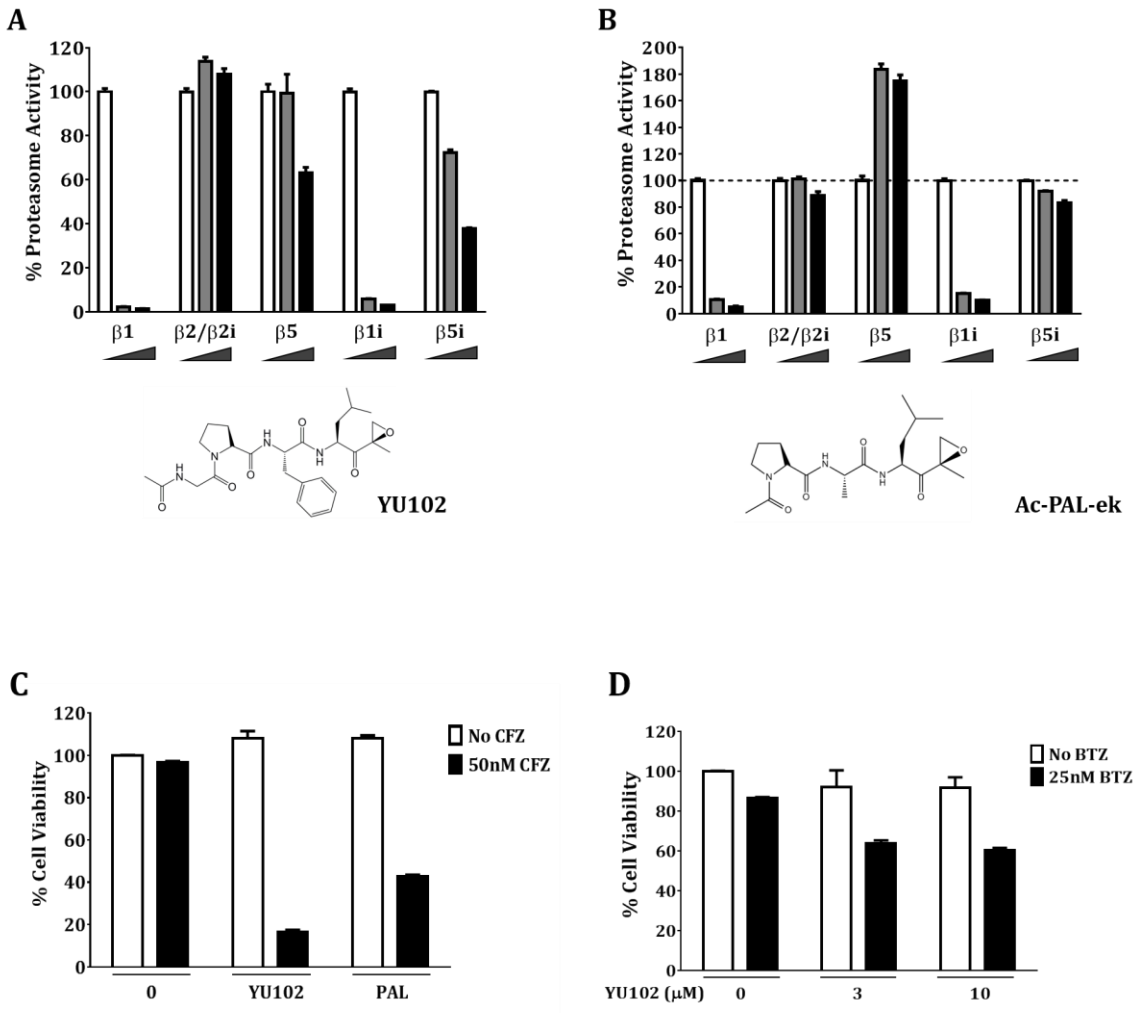


Figure 4.4 β1/β1i-selective proteasome inhibitors YU102 and Ac-PAL-ek sensitize BxPC3/CFZR cells to CFZ.

Proteasome inhibitors YU102 (A) and PAL (B) inhibit β1 and β1i activities selectively. BxPC3/CFZR cells grown in the absence of CFZ for 1 week were treated with 3 μM YU102 or 500 nM Ac-PAL-ek (PAL). Proteasome activities were measured 1 h following treatment with YU102 or PAL. Percent activities are normalized to DMSO control and shown as mean ± SEM. (C) Co-treatment of BxPC3/CFZR cells cultured in the absence of CFZ for 1 week with a non-toxic concentration of CFZ (50 nM) and 3 μM YU102 resulted in enhanced cytotoxic

activity compared to either agent alone. Co-treatment with 500 nM PAL resulted in similar potentiating effects on CFZ activity albeit to a lesser extent compared to YU102. (D) Co-treatment with YU102 (3 and 10 μM) had slight potentiating effects on BTZ activity in BxPC3/CFZR cells. Cell viability was measured at 72h following drug treatment, results are represented as percent viability normalized to DMSO control and shown as mean ± SEM.

4.3.6 PSMB6 Knockdown Potentiates CFZ Activity in BxPC3/CFZR Cells

As a complimentary approach to the chemical inhibition strategy, we also utilized a genetic knockdown approach to further assess the role of the C-L activity of the proteasome in determining CFZ sensitivity. We transfected BxPC3/CFZR cells with siRNA targeting the *PSMB6* gene (siPSMB6) encoding β1 and confirmed knockdown by analyzing β1 activity 48 h following transfection. We observed complete inhibition of β1 activity in BxPC3/CFZR cells transfected with siPSMB6 indicating β1 knockdown (Figure 4.5). Additionally, we also observed decreases in activity levels of β5 and β1i (Figure 4.4=5A), likely due to the cooperative nature of proteasome assembly and feedback regulation of proteasome genes [285]. When challenged with 25 nM and 50 nM CFZ, BxPC3/CFZR cells transfected with siPSMB6 displayed increased sensitivity to CFZ-mediated cytotoxicity compared to BxPC3/CFZR cells transfected with scrambled control siRNA (Figure 4.5B). Similarly potentiating effects were also observed with BTZ and *PSMB6* knockdown (Figure 4.5C).

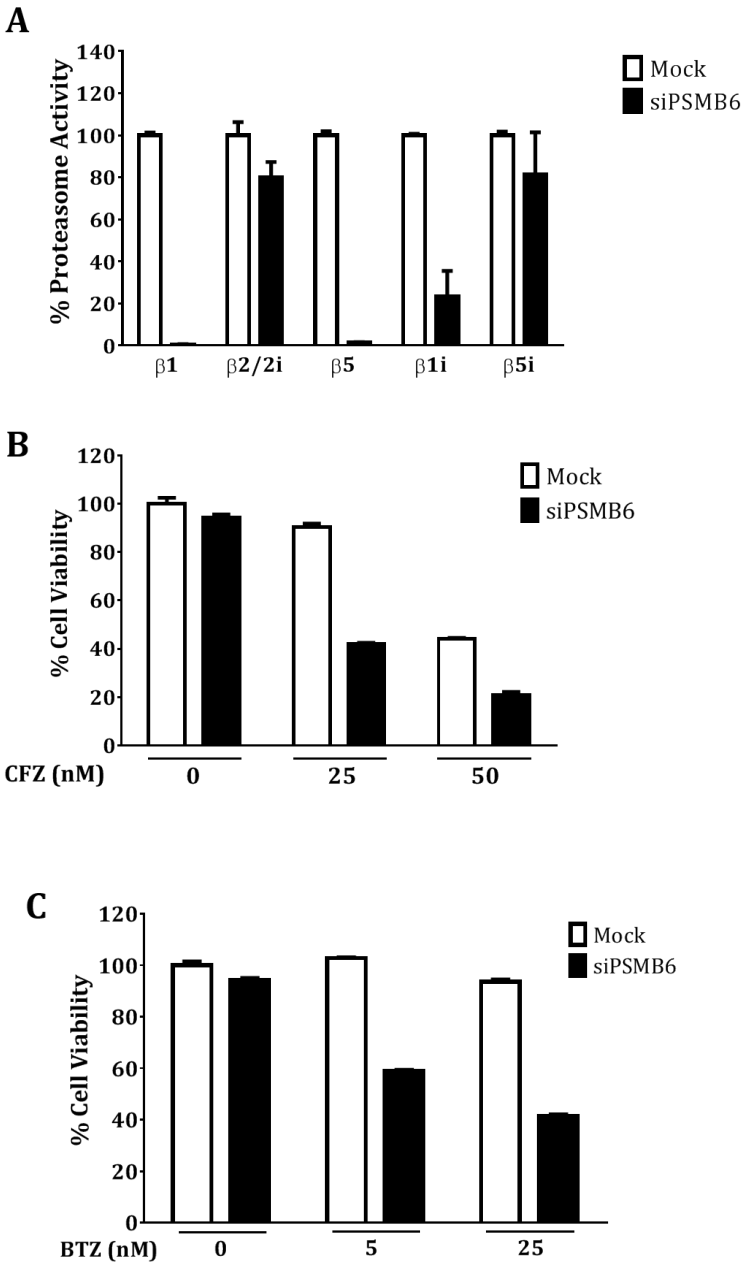


Figure 4.5 PSMB6 knockdown sensitizes BxPC3/CFZR cells to CFZ and BTZ.

(A) Transfection of BxPC3/CFZR cells with PSMB6-targeting siRNA (siPSMB6) markedly decreased β1, β1i, and β5 proteasome activities in BxPC3/CFZR cells. Proteasome activities were measured by selective fluorogenic substrates, results are represented as percent

activity of mock controls and shown as mean ± SEM. (B) siPSMB6 transfection potentiated CFZ cytotoxicity in BxPC3/CFZR cells. siPSMB6 was transfected at 125nmole for 48h followed by 25 nM CFZ treatment. (C) siPSMB6 transfection potentiated BTZ cytotoxicity dose-dependently in BxPC3/CFZR cells. Percent viabilities are normalized to mock control and represented as mean ± SEM.

4.3.7 YU102 Sensitizes RPMI-8226/CFZR Cells to CFZ

In order to examine the relevance of our findings in another model, we established a subline of RPMI-8226 MM cells with acquired resistance to CFZ (RPMI-8226/CFZR). RPMI-8226/CFZR cells were adapted to a final concentration of 30 nM CFZ and exhibited an approximately 36-fold increase in CFZ IC_{50} value compared to RPMI-8226/P cells (Figure 4.6A, IC_{50} values of 269.8 nM vs. 7.4 nM). We did not detect any mutations in the β5-encoding gene, thus ruling out its potential contribution to CFZ resistance in RPMI-8226/CFZR cells. We found that P-gp activity had a contributing role to the CFZ resistance in RPMI-8226/CFZR cells. However, P-gp inhibition by Reversin 121 (P121) [241] only partially restored CFZ sensitivity (Figure 4.6B), suggesting that other mechanisms may also contribute to CFZ resistance in RPMI-8226/CFZR cells. To further explore additional resistance mechanisms, we took a similar approach as described earlier and compared the proteasome activity profile of RPMI-8226/CFZR cells grown in the absence of CFZ for one week to that of RPMI-8226/P cells. We found that RPMI-8226/CFZR cells displayed increased levels of β1, β1i, and β2/2i activities (Figure 4.6B, filled bars) and decreased levels of β5 and β5i activities (Figure 4.6B, blank bars) compared to RPMI-8226/P controls. We then assessed the effect of β1/1i inhibition on CFZ sensitivity in RPMI-8266/CFZR cells using the YU102. Consistent with results in BxPC3/CFZR cells, we found that co-treatment of 3 μM YU102 with 100 nM CFZ (no cytotoxicity alone) led to enhanced cell-killing effects in RPMI-8226/CFZR cells (Figure 4.5C).

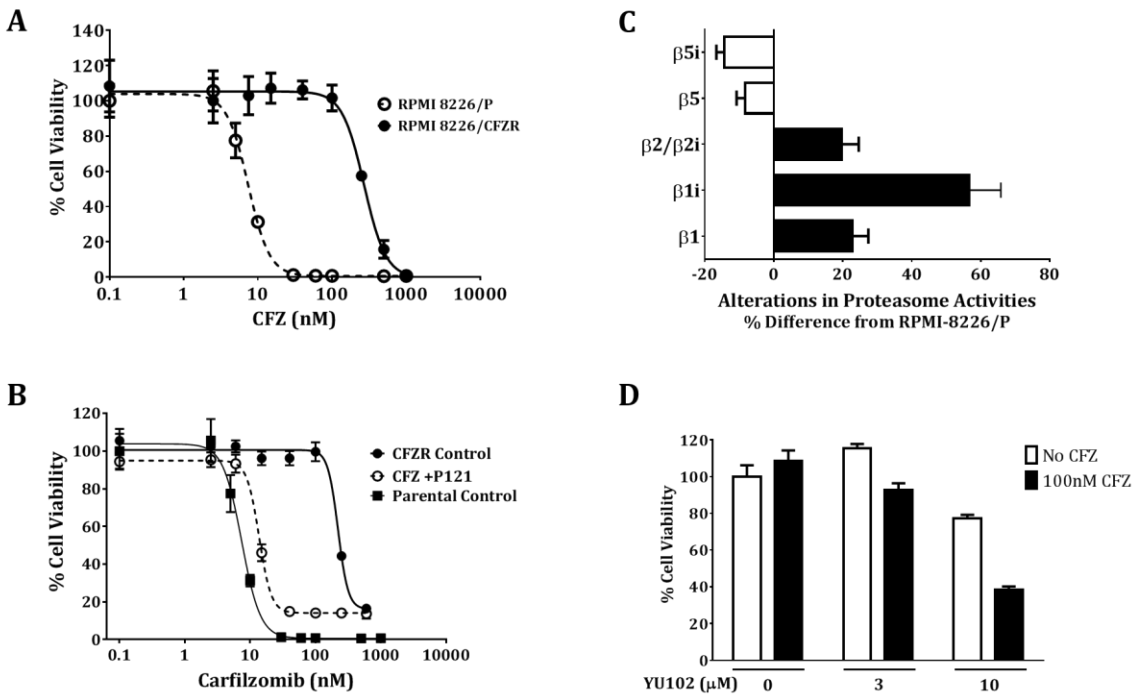


Figure 4.6 Inhibition of C-L proteasome activity sensitizes CFZ-adapted RPMI-8226 multiple myeloma cells to CFZ.

(A) RPMI-8226/CFZR cells display an approximately 36-fold increase in CFZ IC_{50} compared to RPMI-8226/P cells. (B) P-gp inhibition by small peptide inhibitor P121 partially restores CFZ sensitivity in BxPC3/CFZR cells. (C) RPMI-8226/CFR cells exhibited increased β1, β1i, and β2/2i baseline activities compared to RPMI-8226/P cells. Activities were measured in cells grown in the absence of drug for 72h. Results are normalized to each respective activity of RPMI-8226/P cells and shown as percent activity of parental baseline controls. (D) YU102 (3μM or 10μM) potentiated CFZ cytotoxicity dose-dependently in RPMI-8226/CFZR cells. Cell viability was measured 72h following treatment. Results are shown as % cell viability of DMSO control, represented as mean ± SEM.

4.4 Discussion

Drug resistance is a major factor limiting the utility of CFZ in hematological and non-hematological cancers. In order to harness the full therapeutic potential of CFZ, it is essential to enhance our understanding of the mechanisms underlying CFZ resistance. Here, we report a previously unexplored role for the C-L activity of the proteasome in conferring CFZ resistance to cancer cells. In the current study, we observed that CFZ-resistant BxPC3 and RPMI-8226 cells displayed elevated β1 expression and activity, and that β1 activity remained largely uninhibited when challenged by CFZ. We interrogated the role of the C-L proteasome activity in determining PI resistance by using chemical inhibition and genetic knockdown approaches and found that inhibition of the C-L proteasome activity sensitized resistant cells to CFZ. These findings suggest an important role for the C-L activity of the proteasome in cell survival against CFZ-mediated cytotoxicity. The findings reported here provide a rationale for the development of PIs targeting the C-L activity as a potential strategy to improve CFZ efficacy and overcome resistance. Future investigations using *in vivo* and patient-derived models are warranted to determine the clinical utility of targeting the C-L activity of the proteasome.

The current strategy of targeting the C-L proteasome activity to enhance CFZ efficacy is supported by previous studies which suggested that the C-L activity of the proteasome plays a significant role in the survival of cancer cells against proteasome inhibition. For instance, PI-induced upregulation of β1 expression in cells was associated with tolerance to oxidative and proteotoxic stress, while genetic knockdown of β1 enhanced cellular sensitivity to the effects of proteasome inhibition [166, 167]. In general, alterations in the composition of proteasome catalytic subunits have been associated with cellular response to PI [165, 286]. However, the direct impact of such alterations in proteasome expression on the PI sensitivity of cells has not been addressed until recently. In this regard, a recent report demonstrated that selective inhibition of the T-L proteasome activity enhances the cytotoxic effects of BTZ and CFZ against resistant MM cells [169]. These findings were particularly interesting because they provide the first evidence linking PI sensitivity to a non-CT-L activity of the proteasome, as the CT-L activities have generally been recognized as the most important activity mediating the anti-cancer effects of PI drugs [287, 288].

However, no investigations have been carried out to assess the relationship of C-L proteasome activity and PI resistance.

In the current study, we explored how the C-L proteasome activity may contribute to PI resistance in cancer cells. Our findings point to the importance of proteasome activities beyond the CT-L activity in determining cancer sensitivity to PI therapy [169]. However, it should be noted that alterations in the proteasome expression and activity profiles in response to PI may vary in different models of PI resistance. Whereas Kraus et al. reported β2/β2i activity as an important contributor to PI resistance in U266 and MM1S MM models [169], our results suggest a predominant role for the β1/β1i activities in conferring resistance in the BxPC3 and RPMI-8226 models. Currently it is not known how selective alterations in the C-L or T-L activities of proteasomes are achieved in response to PI treatment and further investigations are warranted to understand the regulatory mechanisms involved.

To further interrogate the role of C-L activity in CFZ resistance, we used a chemical inhibition approach. YU102 and PAL were initially developed as inhibitors selectively targeting the β1/1i subunits of the proteasome, but also affected activities of the β 5/5i subunits at high concentrations [57, 284]. This was not entirely surprising as previous studies have shown that proteasome inhibitors utilizing leucine moieties in their P1 positions such as YU102 and PAL may also target β 5/5i subunits at high concentrations [282, 283]. Similar to the chemical inhibition approach, genetically knocking down individual proteasome subunits also has limitations. The cooperative nature of the proteasome assembly process makes it difficult to assess the effect of knocking down an individual subunit without affecting the function of the proteasome as a whole. Additionally, genetically silencing specific proteasome subunits may also trigger a feedback regulatory mechanism of non-targeted proteasome subunits [289, 290]. Despite these experimental limitations, our results appear to consistently support the fact that inhibition of the the C-L activity of the proteasome can enhance the anti-cancer effects of BTZ or CFZ.

In addition to the β1-mediated resistance described in the findings here, we also examined whether previously reported mechanisms of CFZ and BTZ resistance play a role here. We

assessed the status of β5 mutations in our CFZ-resistant cell lines by sequencing and found no mutations present, suggesting that β5 mutation is not relevant in the current CFZ resistance models. Additionally, we examined whether increased P-gp activity contributes to CFZ resistance in the cell line models here by examining the effect of P-gp inhibition on CFZ sensitivity. While we detected no effect in BxPC3/CFZR cells, we found that P-gp inhibition significantly sensitized RPMI-8226/CFZR cells to CFZ, indicating that P-gp activity is a determining factor to CFZ sensitivity in these cells. However, despite the role of P-gp, notable changes in proteasome composition and activities were observed nonetheless, suggesting multiple mechanisms of resistance at play. In line with this, our lab has previously observed alterations in CFZ binding and proteasome expression in other resistant cell lines despite P-gp overexpression, which further support that multiple resistance mechanisms may exist in a given cell line model, and that proteasome-dependent changes may not be mutually exclusive to proteasome-independent alterations in contribution to overall resistance. Consistent with this, results from the present study also showed that inhibition of β1/1i proteasome activities was efficacious in sensitizing both P-gp mediated and non-P-gp-mediated resistant cells.

Our results also showed that IFN-γ-induced alterations to proteasome composition, specifically the upregulation of IP catalytic subunits and concurrent downregulation of constitutive catalytic subunits, sensitized both BxPC3/P and BxPC3/CFZR cells to CFZ and BTZ. These findings were complementary to our results two ways. First, the effects of IFN-γ treatment helped confirm the relevance of the changes in proteasome subunit expression detected in our resistant cell lines to their PI sensitivities. Second, the impacts observed on BTZ and CFZ sensitivities in IFN-γ-treated cells further provided evidence for the importance of CP vs IP activities in in conferring resistance in BxPC3/CFZR cells. Since YU102 and PAL both cross-reacted with multiple CP and IP catalytic subunits, chemical inhibition in this case could not easily differentiate the contributions of β1 and β1i to CFZ resistance. In regards to this, the sensitizing effects of IFN-γ treatment in BxpC3/CFZR cells suggested that upregulation of β1 may be the more dominant resistance-conferring factor in this case. Overall, our findings are also in agreement with previously reported findings which suggested that IP downregulation was responsible for BTZ resistance observed in MM cell lines and patient samples [172]. Furthermore, others have also shown that

induction of IP expression in BTZ- and CFZ-resistant cells can restore PI sensitivity [171]. Taken together, our findings are in support of the notion that IP expression is correlated with PI sensitivity and suggest that baseline IP expression may be used as a predictor of PI response.

The overall outcomes of both chemical and genetic knockdown approaches in the current study provide further basis for the combined inhibition of CT-L and C-L proteasome inhibition as a potential strategy to overcome PI resistance. These findings provide a rationale for the design and development of future proteasome inhibitors with more general inhibitory activities toward multiple catalytic sites of the proteasome which may yield superior anti-cancer efficacy compared to CT-L-selective compounds. Our results further support that overall cellular sensitivity to PI agents may be determined by the activities and possible interactions of multiple proteasome catalytic subunits. Thus, it may be necessary to more carefully evaluate how the modulation of multiple proteasome activities may improve the overall anti-cancer efficacy of PI therapy. Going forward, validating the effect of C-L inhibition *in vivo* and in clinically-relevant models are necessary to better understand the translatability and therapeutic potential of this approach. Optimization and further characterization of proteasomal-dependent alterations in patient-derived CFZ-resistant cells will also be important to further demonstrate the prevalence and relevance of C-L activities in clinical CFZ resistance.

4.5 Conclusion

In summary, we report that the upregulation of the C-L activity of the proteasome may contribute to CFZ resistance, and that co-inhibition of the CT-L and C-L activities may overcome CFZ resistance in pancreatic cancer and MM cells. Findings presented here provide basis for the development of new PIs targeting the C-L activity as strategies to overcome CFZ resistance. Such investigations may be important in providing new therapeutic options for cancer patients with PI resistance.

Chapter 5 POLYMER MICELLE FORMULATIONS OF PROTEASOME INHBITOR CARFILZOMIB FOR IMPROVED METABOLIC STABILITY AND ANTI-CANCER EFFICACY IN HUMAN MULTIPLE MYELOMA AND LUNG CANCER CELL LINES

The work in this chapter has been published in the Journal of Pharmacology and Experimental Therapeutics 2014 355(2): 168-73 [291]. Permission to publish in the current dissertation was obtained from Journal of Pharmacology and Experimental Therapeutics.

5.1 Introduction

The proteasome is a multimeric protease complex that is central to the highly-regulated ubiquitin-proteasome protein degradation system [292]. The proteasome plays a key role in regulating numerous signaling pathways involved in cell proliferation, cell cycle control, and apoptosis, which are often found to be dysregulated in malignant cells [2, 293]. During the past decade, proteasome inhibition has proven to be an effective anti-cancer strategy with the FDA approval and revolutionary success of the first-in-class proteasome inhibitor agent BTZ (Velcade®, BTZ) in the treatment of multiple myeloma [294]. However, BTZ therapy has several drawbacks including dose-limiting neurotoxicity that is likely due to off-target interactions of its boronic acid pharmacophore [295, 296]. This issue was addressed with the approval of a second-generation proteasome inhibitor CFZ (Kyprolis®, CFZ). CFZ is a tetrapeptide equipped with a C-terminal epoxyketone warhead that irreversibly interacts with the active site of the proteasome in a more selective manner than BTZ [8]. CFZ has demonstrated efficacy in both BTZ-naïve and BTZ-resistant patients, and possesses a more favorable toxicity profile compared to BTZ [13, 14, 297]. With these improvements, CFZ along with lenalidomide and dexamethasone has been recently shown to provide unprecedented benefit in patients with multiple myeloma [131].

Due to its promising anti-cancer activities and favorable toxicity profile, CFZ has also been explored as a potential therapeutic for malignancies other than multiple myeloma. Results from several investigations indicated that CFZ has potent cell-killing activity toward various solid cancer cell lines [6, 298, 299]. Based on these promising preclinical findings, a Phase

I/II study was initiated to further assess the therapeutic potential of CFZ in patients with advanced solid cancers. Disappointingly, CFZ demonstrated little to no anti-tumor activity clinically [17]. Although the exact mechanisms underlying the discrepancies between the *in vitro* and clinical data are currently unknown, one potential explanation is the rapid metabolic degradation of CFZ *in vivo* [136]. CFZ degradation in humans is mainly due to peptide cleavage and epoxide ring opening, resulting in plasma half-life of less than 30 min [137]. We postulated that the fast metabolic inactivation of CFZ in the body might hinder the ability of the active drug to accumulate in solid cancer tissues, leading to insufficient target inhibition and poor clinical efficacy. Thus, increasing the metabolic stability of CFZ may serve as a strategy to improve its overall anti-cancer efficacy.

Another major challenge in delivering CFZ *in vivo* is its poor water solubility. Currently, this problem is addressed by complexing a 60 mg dose of CFZ with 3,000 mg of sulfobutylether β-cyclodextrin (SBECD) (FDA prescribing information). SBECDs interact with hydrophobic portions of drugs to reduce their interactions with the environment, thus increasing their solubility [300]. SBECD was however shown to offer little protective effect against metabolic degradation of CFZ *in vivo* [137]. Thus, it would be useful to develop alternative CFZ formulations that can improve its metabolic stability in addition to solubility. Our current study explored the utility of polymer micelles in improving the metabolic stability of CFZ against enzyme-mediated degradation and delivering CFZ in a controlled manner. Polymer micelles are highly efficient in entrapping hydrophobic drug molecules inside the core and prevent the drugs from precipitating, binding to serum proteins, or being degraded by enzymes in the body [301]. Polymer micelles may also be effective in increasing accumulation of anticancer drugs in solid tumors by passing through the leaky blood vessels near tumors and sparing healthy organs with well-organized blood vessels [302]. Additionally, polymer micelles are readily modified chemically or with attachment of surface ligands to control drug release patterns as well as cell-targeted drug delivery, making them versatile vehicles for delivery [303]. Previous studies have shown that polymer micelles significantly reduced toxicity and improved anticancer activity of chemotherapeutics by achieving sustained drug release and increasing drug exposure to cancer cells [304, 305].

In our current study, we prepared micelle particles composed of biodegradable block copolymers poly-(ethylene glycol) (PEG) and poly-(caprolactone) (PCL), both of which are generally recognized as safe by the FDA. PEG is a hydrophilic polymer that can improve the solubility as well as increase the circulation time of the particles inside the body [306]. PCL provides the hydrophobic platform with which CFZ molecules can readily interact, allowing for efficient drug encapsulation [307]. We prepared six PEG-PCL-based micelle formulations with varying molecular weights of the PEG-PCL block to increase drug loading and with calcium phosphate (CP) or deoxycholic acid (DCA) excipients introduced to stabilize the core of the polymer micelles. To simplify the polymer micelle formulation, we used PEG end-capped with a methoxy group in our study.

Here, we report our results demonstrating the potential of polymer micelle formulations of CFZ in improving metabolic stability and thereby extending therapeutic applicability of CFZ. These results may serve as the foundation for further optimization of CFZ formulations and investigations of their potential benefits for the treatment of various types of cancers.

5.2 Materials and Methods

5.2.1 Cell Lines and Reagents

PEG N-hydroxysuccinimide ester was obtained from NanoCS (Boston, MA). Branched poly(ethylene imine) (25,000 molecular weight), palmitoyl chloride, HEPES buffer, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), diethyl ether, dimethyl formamide and ethanol were purchased from Sigma Aldrich (St. Louis, MO). PEG-PCL block copolymers with molecular weight 5,000-2,300 (PEG-PCL 5-2) or 5,000-5,500 (PEG-PCL 5-5) were purchased from Polymer Source (Montreal, CA). CFZ was purchased from LC laboratories (Woburn, MA). All other reagents used in the metabolism studies were purchased from Sigma-Aldrich (St. Louis, MO). Established human cancer cell lines derived from lung (H460) and B-lymphocytes (RPMI-8226) were purchased from American Type Culture Collection (ATCC) and maintained according to ATCC recommended conditions. All other reagents were obtained from Fisher Scientific (Waltham, MA) unless mentioned otherwise.

5.2.2 Preparation of CFZ-loaded polymer micelles

We prepared a total of six CFZ-loaded polymer micelle formulations in this study as summarized in Table 1: CFZ-loaded micelles, prepared from PEG-PCL 5-2 or PEG-PCL 5-5, with CP, DCA, or no excipient. In a 50 mL round-bottom flask, 1 mL CFZ (1 mg/mL ethanol) and 100 µL PEG-PCL (100 mg/mL ethanol) stock solutions were mixed at 60°C in the presence of additives: 20 µL ethanol was added for excipient-free micelles, 20 µL Na_2HPO_4 (10 mg/mL in water) for CP-containing micelles, and 20 µL DCA (10 mg/mL in ethanol) for DCA-containing micelles. Ethanol was evaporated under reduced pressure by using a rotatory evaporator to create a thin film at the bottom of each flask. The thin film was rehydrated with deionized water and gently mixed to allow PEG-PCL to self-assemble into polymer micelles entrapping CFZ. For CP-containing micelles, 20 µL $CaCl_2$ (10 mg/mL water) was added in this step to prepare CP-containing micelles. The flask was subsequently sonicated for 5 min, and the solution was transferred to a conical tube and centrifuged to remove insoluble free drug, insoluble excipients, and other impurities. The supernatant containing CFZ-loaded micelles was collected and divided into tubes for freeze-drying. Freeze-dried micelles were weighed and stored at -20°C until use. The extent of drug

loading was determined by mass percent composition of CFZ in total CFZ-loaded micelles (w/w %). We quantified CFZ in micelles by high performance liquid chromatography (HPLC, Shimadzu LC20 system, Agilent XDB-C18 column equipped with a photo diode array detector (SPD-M20A using a mobile phase of H_2O:CH_3CN with 0.1% formic acid (45:55, v/v) at a flow rate of 1 mL/min, 40°C)). Encapsulation efficiency was defined as the percent of drug encapsulated to the drug added. Drug loading efficiency was defined as the weight percent of drug encapsulated to the weight of polymer added. According to the CFZ w/w %, we reconstituted freeze-dried micelles in water or buffer solutions and serial dilutions were made to prepare CFZ concentrations for experiments described below.

5.2.3 In vitro metabolism of CFZ polymer micelles in mouse liver homogenates

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Whole livers were harvested from five female C57BL/6J mice (8 weeks old, liver weight 1.203 g-1.431 g) and washed three times with ice-cold PBS (pH 7.4) in petri dishes. Livers were cut into small pieces and homogenized with a 15 mL glass dounce homogenizer (Kimble Glass) in a 1:5 volume of ice-cold PBS. Liver homogenates (200 mg/mL) were pre-incubated at 37°C for 1 min before the addition of free CFZ or micelle formulations of CFZ (final CFZ concentration of 1 μM). An aliquot of 40 μL of reaction mixture was taken at 0, 5, 10 and 20 min at 37°C and quenched with 120 μL cold acetonitrile containing phenytoin (0.5 μM, an internal standard) and kept on ice for 30 min, followed by centrifugation at 16,100*g* for 15 min at 4°C. The concentrations of CFZ in the supernatants were measured using an Agilent 1200 HPLC interfaced with Agilent 6410 Triple Quadrupole tandem mass spectrometry (MS/MS) equipped with an electrospray ion source. Briefly, chromatographic separation was carried out with a Waters XTerra MS C18 column (2.1×50 mm, 3.5 μm; Waters Corporation, Milford, MA). Mobile phase was delivered at 250 μL/min, and the gradient was initiated at 90% A-10% B [A, 0.1% (v/v) aqueous formic acid; B, acetonitrile]. The proportion of mobile phase B was increased to 90% over 1 min, held constant for 2 min, and then restored to the initial composition. Following the injection of 10μL samples, CFZ and phenytoin were detected by MS/MS spectra obtained in the positive ion mode; CFZ by detecting the transitions 720.4 → 402.2 m/z and phenytoin by detecting the transitions 253.2 → 182.2 m/z. All data were acquired employing Agilent

6410 Quantitative Analysis software.

5.2.4 In vitro CFZ release profiles of polymer micelle formulations

Each formulation was dissolved in phosphate-buffered saline (PBS) to an equivalent CFZ concentration of 100 μM. For each sample, 100 μL of polymer micelle formulation was added to five dialysis cups and dialyzed against 1 L of PBS at 37 °C. A sample of 35 μL of each formulation was removed from the dialysis cups at 0, 1, 3, 6, 24, 48 and 72 h. CFZ concentration was analyzed by HPLC equipped with a photo diode array detector described above. Percent changes in CFZ concentrations were obtained by normalization to the value obtained at 0 h for each formulation. Drug release profiles for each formulation were analyzed by assuming second-order release kinetics, and by calculating the area under the curve (AUC) values.

5.2.5 Cell Viability Assay

H460 and RPMI-8226 cells were seeded in 96-well plates at 5,000 and 10,000 cells per well, respectively. Following overnight incubation, cells were treated with free CFZ solution or one of the six CFZ micelle formulations at various concentrations for 72 h. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega) following manufacturer's protocol. Relative cell viability was obtained from arbitrary luminescence units by normalization to drug-naïve controls. Statistical analysis was carried out using GraphPad Prism (GraphPad Software). One-way ANOVA was used to compare multiple groups and $p<0.05$ was deemed to be statistically significant.

5.3 Results

5.3.1 Preparation of polymer micelle particles

Our initial goal was to identify a polymer micelle formulation that will allow us to achieve improved metabolic stability of CFZ. We prepared six polymer micelle formulations of CFZ composed of PEG-PCL block copolymers with identical 5000 g/mole PEG portions and varying PCL portions to maximize drug loading (Table 5.1). Micelles containing short (2,300 g/mole) or long (5,500 g/mole) PCL portions are designated as PEG-PCL 5-2.3 (PM1) and PEG-PCL5-5 (PM2), respectively. We also incorporated excipients calcium phosphate (CP) or deoxycholic acid (DCA) into PM1 or PM2 formulations, with the goal of improving overall stability of the micelle particles. Formulations with the added CP or DCA were found to have increased weights, consistent with successful incorporation of these excipients into the micelle particles. To ensure the final particles contained mainly fully incorporated particles, insoluble CP or DCA was removed by subsequent centrifugation of reconstituted micelle solution. The drug loading efficiencies of micelle particles with differing sizes of the PCL portions and different excipients varied in the following order: PM1-DCA > PM1 ≈ PM1-CP > PM2-CP > PM2-DCA > PM2 (Table 5.1).

5.3.2 Polymer micelle formulations improve CFZ metabolic stability to varying extents in vitro

The metabolic stability profiles of the six CFZ-containing micelle formulations were compared against free CFZ solution by measuring the rate of CFZ disappearance in the presence of mouse liver homogenates (Fig 5.1). Our results showed that free CFZ rapidly disappeared in the presence of mouse liver homogenates, with less than 10% of the active drug remaining in 10 min of incubation. In contrast, all six micelle formulations of CFZ had improved stability profiles, demonstrated by at least 50% of the active CFZ remaining after 10 min of incubation (Fig 5.1). Among the six different micelle formulations, PM1-CP particles demonstrated the least protective effect against CFZ degradation in the presence of mouse liver homogenates. All other micelle formulations displayed similar protective effects against the metabolic degradation of CFZ. Similar results were also obtained using human liver microsomes.

Table 5.1 Description of the six polymer micelle formulations of CFZ and their respective drug loading and encapsulation efficiencies.

Formulation	Description	Drug Loading Efficiency (%)	Encapsulation Efficiency (%)
PM1	PEG-PCL 5-2.3 kD	4.0	40.0
PM1-CP	PEG-PCL 5-2.3 kD, calcium phosphate	3.9	38.6
PM1-DCA	PEG-PCL 5-2.3 kD, deoxycholic acid	4.4	44.3
PM2	PEG-PCL 5-5.5 kD	1.0	9.9
PM2-CP	PEG-PCL 5-5.5 kD, calcium phosphate	3.1	31.0
PM2-DCA	PEG-PCL 5-5.5 kD, deoxycholic acid	2.3	23.4

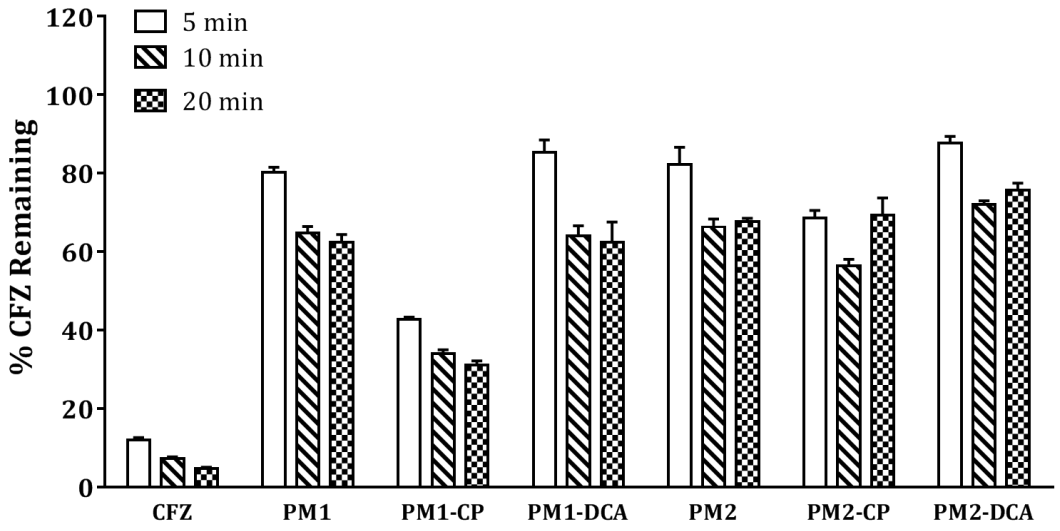


Figure 5.1 *In vitro* metabolic stability of polymeric micelle formulations containing CFZ in the presence of mouse liver homogenates.

CFZ remaining was measured at 0, 5 (blank bars), 10 (striped bars), and 20 min (checkered bars) following incubation with liver homogenates. Percent CFZ remaining values were normalized to 0-min control, and results are represented as means ± SEM. All nanoparticle groups differ from CFZ control determined by One-Way ANOVA analysis, with $p<0.05$.

5.3.3 Polymer micelle formulations exhibited varying rates of CFZ release over 72 hours

Next, we characterized the CFZ release profiles of the micelle particles *in vitro* by measuring the rate of drug release from the particles over 72 h. All six formulations demonstrated rapid CFZ release in the first 20 h followed by a slower, sustained release for up to 72 h (Fig 2). We fit the data to a two-phase decay model and obtained kinetic parameters for the fast- and slow-release phases (Table 5.2). The $t_{1/2}$ values during the fast-release phase were similar among all six particles, averaging around 1 h. In comparison, the slow-phase release profiles differed substantially among the six formulations, with PM1, PM2-CP, and PM2-DCA being the slowest-releasing formulations (Table 5.2). We also analyzed CFZ release from the polymer micelles by comparing area under the curve (AUC) over 72 hours. AUC analysis is a model-independent analysis method that allows for direct comparison of release profiles. In the current study, the larger AUC values the formulations have, the more CFZ would remain with polymer micelles over time. Our AUC analysis results showed that PM1, PM1-DCA, and PM2-DCA had the highest AUC values, corresponding to slower drug release, whereas PM1-CP, PM2 and PM2-CP had low AUC values, corresponding to faster drug release rates (Table 5.2).

5.3.4 Polymer micelle formulations display comparable or improved anti-cancer activities compared to free CFZ

When tested using human lung cancer cell line H460, we found that four of the polymer micelle formulations had more potent cell-killing effects compared to free CFZ solution (Fig 5.3A). To exclude the possibility that components of the micelle particles themselves contributed to the overall cytotoxicity, we also measured the cell-killing effects of empty micelle particles of both molecular weight compositions in H460 cells. Our results indicated that micelle particles without CFZ entrapment did not possess any cytotoxic effects (Fig 5.4A: PM1 Empty, PM2 Empty). As another control, we also measured the effect of the physical mixture of empty particles and free CFZ on H460 cell viability. No statistically significant difference was observed between the viabilities of cells treated with the physical mixture of particles and CFZ compared to those treated with free CFZ alone (Fig 5.4A: PM1 Empty + free CFZ, PM2 Empty + free CFZ). Similar results were obtained using a human multiple myeloma cell line, RPMI-8226 (Figs 5.3B & 5.4B).

Table 5.2 Kinetic parameters of *in vitro* CFZ release from six polymer micelle particles based on two-phase decay modeling and AUC analyses

Formulation	$t_{1/2}$ fast (h)	$t_{1/2}$ slow (h)	AUC (µmol/L*h)
PM1	1.3 ± 0.7	36.0 ± 13.7	2372
PM1-CP	0.8 ± 0.3	18.8 ± 5.1	1346
PM1-DCA	1.0 ± 0.4	25.0 ± 12.5	2195
PM2	0.7 ± 0.2	26.1 ± 8.0	1419
PM2-CP	1.4 ± 0.4	36.4 ± 22.4	1160
PM2-DCA	0.1 ± 0.1	39.2 ± 10.0	3229

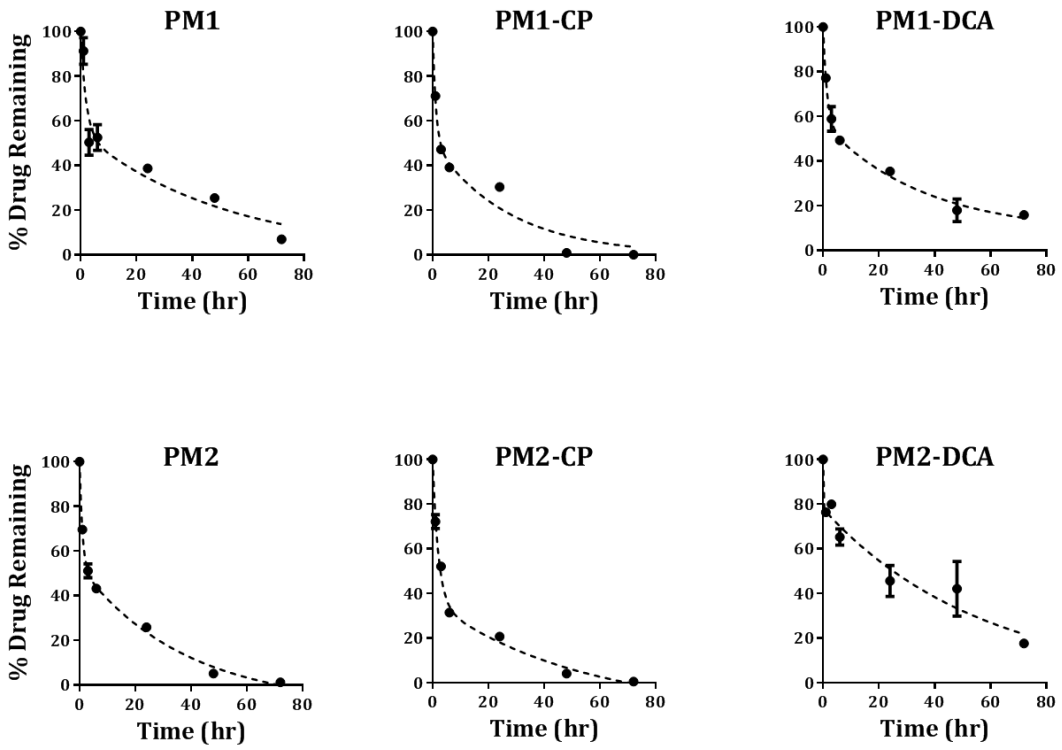


Figure 5.2 *In vitro* drug release profiles of polymeric micelle formulations.

CFZ release was measured over 72 h and represented as percent drug remaining of control. Results are represented as means ± SEM and each data set is fitted to a two-phase decay model (dotted curve).

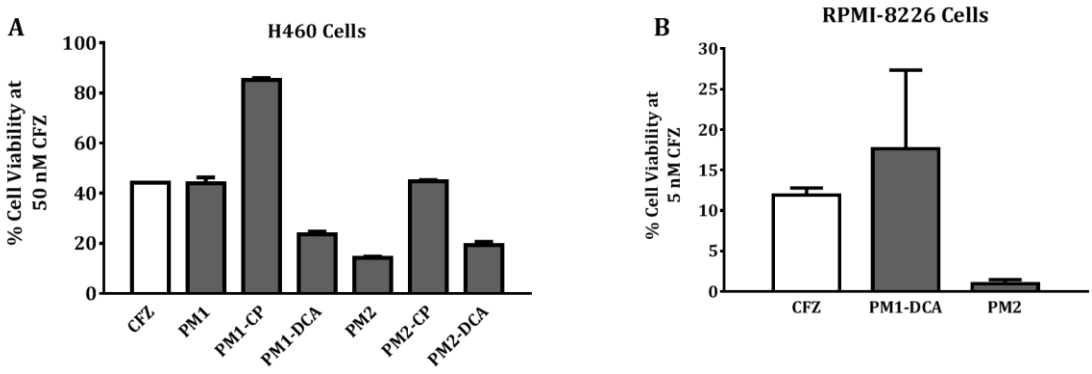


Figure 5.3 Cytotoxic activities of polymer micelles containing CFZ in H460 and RPMI-8226 cells.

Cytotoxic activities of polymer micelles containing CFZ in H460 (A) and select micelle formulations in RPMI-8226 (B) cell lines. Results are represented as percent cell viability of vehicle-only control. Micelle formulations containing CFZ were compared at 50 nM and 5 nM of equivalent CFZ concentration in H460 and RPMI-8226 cells, respectively. Data is represented as means ± SEM.

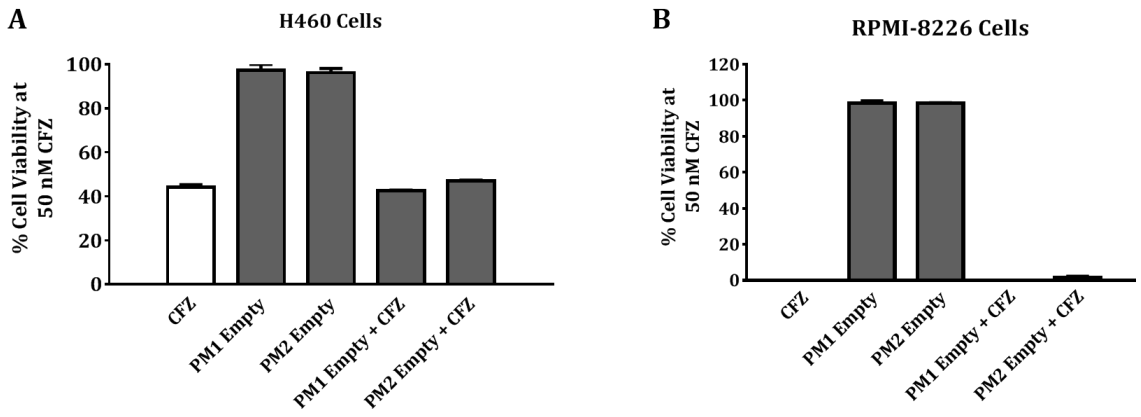


Figure 5.4 Cytotoxic effects of PM1 and PM2 empty particle controls and co-incubation of CFZ with empty particles tested in H460.

Effects of empty polymer micelles on cell viability of H460 (A) and RPMI-8226 (B) cells. Results are represented as percent cell viability of vehicle-only control. Empty particle controls and co-incubation controls were compared at 50 nM equivalent CFZ concentration in both cell lines. Data is represented as means ± SEM.

5.4 Discussion

In the current study, we assessed the potential of polymer micelle formulations to improve metabolic stability and overall anti-cancer efficacy of the proteasome inhibitor CFZ. Our results demonstrated that CFZ-loaded PEG-PCL polymer micelles were more metabolically stable than free CFZ solution, supporting an extended circulation of the active drug *in vivo*. In addition, several of our micelle formulations of CFZ demonstrated potential to enhance anti-cancer activity compared to free CFZ solution. The current study is the first to report on the feasibility of polymer micelles in improving the delivery of proteasome inhibitor agents. Further investigations are warranted to examine the utility of these new formulations in improving the antitumor activity of CFZ *in vivo* for the treatment of solid cancers by examining metabolic stability, target modulation, and tumor accumulation in preclinical animal models.

Several other studies have previously explored similar strategies of utilizing nanoparticle systems to deliver proteasome inhibitor agents. For example, Ashley *et al* demonstrated improved efficacy and reduced toxicity of VLA-4 targeted liposomal CFZ particles in multiple myeloma cell lines and xenograft models [308]. Similarly, Swami *et al* used a targeted liposomal approach to deliver BTZ to the bone microenvironment [309]. Although the latter study did not achieve direct enhancement in BTZ efficacy, their results supported the utility of nanoparticle systems by demonstrating successful delivery to the targeted bone tissues. Findings from both of these studies support the promising utility of nanoparticle-mediated delivery of proteasome inhibitors by demonstrating the potential therapeutic advantages over the currently existing system.

Unlike the previous studies, which focused on PEGylated liposome delivery systems, we explored the capabilities of a much simpler micelle-based system to serve our goals. There are several advantages to micelle-based delivery over liposome-based platforms, including better efficiency in the loading, carrying, and releasing of hydrophobic drug molecules [302]. Furthermore, micelles have been shown to have better tumor infiltrating abilities, likely due to their smaller sizes compared to liposomes [310, 311]. However, as with any nanoparticle delivery systems, polymer micelles have drawbacks associated with their use in drug

delivery. One of the major concerns with utilizing micelle-based delivery systems is their lack of stability *in vivo* due to disintegration of the particles in the body. In this regard, previous studies have demonstrated that PEG-PCL-based polymer micelle particles carrying the anti-cancer agent paclitaxel exerted more potent anti-cancer activity in a mouse xenograft model than taxol alone [305]. These results suggest that PEG-PCL-based micelles are structurally stable enough *in vivo* to reach the tumor sites.

We have made efforts to address concerns with micelle stability in our current study by including two PEG-PCL micelle particles with the added excipients CP and DCA. Our goals by incorporating such additives into our particles were to further stabilize micelle core structures and to control drug release. CP is a main biological component of bone and teeth, which has been investigated as a surface modification due to its biocompatibility and rigidity to improve the stability of the hydrophobic drug-loaded nanoparticle cores [312]. In solution, calcium ions and phosphate ions react to form solid calcium phosphate. Micelles or other nanoparticles can act as nucleation sites for this reaction, which results in the formation a mineralized surface on the nanoparticle core [313]. CP formed away from the micelle particles can be removed easily, due to the insolubility of CP. DCA is a sterol-based bile acid that acts as an endogenous emulsifying agent by forming micelles to aid in interactions with insoluble compounds inside the body. The addition of sterol compounds to micelles has been previously shown to improve micelle stability and *in vivo* delivery [314], as well as reduce drug release rates [315].

As discussed previously, rapid CFZ metabolism in the body may lead to an insufficient accumulation of active drug in distal tumor sites. To better understand the relationship between the interactions of CFZ with the polymer micelle particles and the overall metabolic stability profile, we characterized the release of CFZ from each particle over time. Assuming drug release is dependent on the concentration of the drug inside the particles relative to its surroundings, measuring drug release can help us approximate the strengths of interactions between CFZ and the varying micelle core environments. All six of the polymer micelle formulations demonstrated sustained CFZ release over 72 hours in rapid- and slow-release phases. Differences in the rate of release were observed mainly in the slow-release phases, which likely derived from stronger and more specific interactions

between CFZ and the polymer micelle particles. Based on our results, both PM1 and PM2 were able to release CFZ in a sustained manner, suggesting that the difference in PCL content may not be the dominant factor in controlling CFZ release. Furthermore, PM1-DCA and PM2-DCA appeared to release CFZ more slowly compared to polymer micelles that did not contain DCA, suggesting that interactions between DCA and CFZ may play a role in slowing CFZ release from micelle particles.

Our findings indicated that both the release rate and the metabolic profile of CFZ may be important in determining the overall efficacy of CFZ, but not with a straightforward relationship between rate of CFZ release and metabolic degradation. We found that PM1-CP and PM2-CP, which released CFZ quickly, had the least anti-cancer activities compared to other formulations. On the other hand, PM1-DCA and PM2-DCA, which had slower CFZ release, achieved better anti-cancer activity. These observations suggest that the rate of drug release plays a role in determining the overall efficacy of the drug, and that micelle particles with slower CFZ release may be better in inducing cancer cell death. In addition to having the quickest release profile, PM1-CP also demonstrated least *in vitro* metabolic stability among the six formulations. This may be an effect of the fast release of CFZ or instability in the structure of the particles that resulted in the breakdown of CFZ and thus poor efficacy in cells. PM2 particles were most effective in inducing cell death among all formulations, despite also possessing a fast CFZ release profile similar to that of PM1-CP. Interestingly, PM2 particles had good metabolic stability despite having quick CFZ release rates, suggesting that metabolic stability may depend on factors other than the rate of drug release. This is also consistent with the fact that differences observed in CFZ release in our study did not necessarily correspond to those observed in metabolic stability profiles. To better understand the necessary balance between drug stability and release rate, further optimizations in particle design and more in-depth assessments of metabolic stability and drug release *in vivo* will be necessary.

In our current study, we compared six polymer micelle formulations that displayed substantial protection of CFZ molecules from degradation in the presence of liver homogenates. The excipients CP or DCA added with an intent to stabilize the micelle core appeared to have an impact on drug release profiles. For example, PM1-DCA and PM2-DCA

appeared to release the drug more slowly than polymer micelles without DCA added (Fig 2 and Table 2). It is plausible that additional favorable hydrophobic interactions between DCA and CFZ may slow down the drug release from polymer micelles. Interestingly, there was no apparent correlation between the extent of protective effects against metabolism and the rate of drug release *in vitro*. To better understand the relationship between drug-micelle interactions and metabolic stability, further investigations with a more extensive library of micelle structures may be necessary.

5.5 Conclusion

Here, we report that polymer micelle formulations of CFZ can improve the metabolic stability and achieve sustained release *in vitro*. Additionally, these formulations show comparable or enhanced anti-cancer activity *in vitro* as free CFZ solution against lung cancer and multiple myeloma cell lines. Findings from this study provide proof-of-concept support of the potential advantages of polymer micelle-mediated delivery over the currently existing formulation system for CFZ. Further investigations of polymer micelle-mediated CFZ delivery in *in vivo* models are necessary to confirm such benefits and to provide further evidence towards potential clinical utility.

Chapter 6 SUMMARY AND FUTURE DIRECTIONS

Drug resistance is a critical problem for nearly all chemotherapies, including PI-based therapies. Currently, there are no available strategies in the clinic to combat PI-resistance due to a lack of knowledge regarding how resistance develops and the factors involved in determining PI response. In order to address this problem, extensive effort has been put forth over the last decade toward improving our understanding of the mechanisms responsible for PI resistance. Of the approved PI agents, resistance to BTZ has been the most extensively studied. At the beginning of our studies in 2011, a few mechanisms had been proposed for BTZ resistance but none was known for CFZ resistance.

Following the initial FDA approval of CFZ in 2012, additional clinical findings were reported indicating its superior efficacy and toxicity profiles, which quickly propelled further expansions to its clinical indications in MM therapy. As CFZ gained recognition for its potent anti-cancer activity and favorable toxicity profile, many researchers also explored its therapeutic utility in other cancer types. However, these efforts were met with further drug resistance challenges. Whereas hematological cancers, especially MM, are highly sensitive to proteasome inhibition, solid cancers were found to be intrinsically resistant. With the reasons underlying this difference in the PI sensitivities of hematological and non-hematological malignancies largely unknown, advancements in developing novel PI-based therapies for solid cancer treatment were halted.

With these challenges in mind, we decided to investigate the molecular factors involved in determining CFZ sensitivity using *in vitro* models. We utilized both hematological and non-hematological cell lines in our studies in order to cross-validate the mechanisms in both contexts. Findings from this dissertation work address both proteasome-dependent and proteasome-independent mechanisms and propose three different strategies to circumvent CFZ resistance. Collectively, our findings support a multi-pronged model of CFZ resistance, where more than one dominating mechanism is likely involved in mediating CFZ resistance as well as cross resistance to BTZ. Further validation of our results will be necessary to determine whether these mechanisms are impactful *in vivo* and clinically.

Our study describing the role of P-gp in mediating acquired resistance to CFZ was one of the first reported suggesting P-gp-mediated drug efflux as a major contributor to determining CFZ response. Since then, several other studies have noted similar observations of P-gp upregulation in other CFZ-resistant cell lines as well as a CFZ-refractory patient. We are encouraged as P-gp continues to be validated in more clinically relevant settings. Going forward, comparative analyses of P-gp expression in the MM cells from a greater number of CFZ-sensitive and CFZ-resistant patients would be helpful to better determine the clinical importance of P-gp upregulation in CFZ response. Furthermore, since P-gp is a known contributor to cancer resistance of many other chemotherapeutics, it would also be important to examine whether P-gp-mediated cross-resistance occurs between PIs and other anti-cancer agents in the clinic. Additionally, our findings demonstrated that small dipeptide derivatives of CFZ can effectively restore CFZ sensitivity in resistant cells. However, in order to confirm that the resistance-reversing effects of the small peptide analogs are the effect of P-gp inhibition, additional experiments will be needed. For instance, assessing the effect of P-gp knockdown on cellular sensitivity to CFZ alone and in combination with the small peptide analogs can further address whether P-gp is the sole target of the small peptide analogs.

Our current study also lacked evidence for the mode of inhibition through which CFZ and the small peptide analogs interact with P-gp. It would be important to include future experiments such as determining the enzyme kinetic parameters of CFZ transport by P-gp and other efflux transporters, as well as assessing how the small peptide analogs affect CFZ transport by P-gp. Such information may provide important insights into the design and further optimization of P-gp inhibitors with improved potency and specificity. Since the small peptide-based CFZ derivatives described here are much smaller in size than most other peptidomimetic P-gp inhibitors, it is possible that they may have more favorable toxicity profiles *in vivo* compared to bulkier peptides. As we only synthesized a small library of CFZ analogs in the proof-of-concept study described in this dissertation work, further optimizations will be necessary to improve their P-gp inhibitory potencies. As well, *in vivo* evaluations will be necessary to determine the specificity of P-gp inhibition and overall toxicity of these compounds.

During our investigations of P-gp-mediated CFZ resistance, it became evident that while P-gp upregulation was the major mechanism mediating CFZ resistance in some cell lines, it was not the only mechanism. In order to investigate other mechanisms involved in conferring acquired CFZ resistance, we established another resistance model that was P-gp-independent. The findings described in chapter 4 suggested a role for the C-L activity of the proteasome in the development of CFZ resistance, potentially by serving as a compensatory mechanism to inhibition of the CT-L activities. These observations are in agreement with previously reported roles of non-CT-L catalytic subunits in determining cellular sensitivity to oxidative stress and PI-induced cytotoxicity. Additionally, findings from this study support the development of subunit-selective proteasome inhibitors, in particular PIs targeting the C-L and T-L activities, as potential therapeutics to enhance BTZ and CFZ activities. Such inhibitors, especially C-L inhibitors, are desirable as inhibition of the C-L activity has been shown to have no cytotoxic effects on its own. Furthermore, our group and others have found that combined inhibition of the C-L and CT-L activities can achieve better anti-cancer efficacy. These findings provided the basis for additional studies investigating the therapeutic potentials of co-targeting the C-L activity. As well, development of PIs with improved selectivity for β1 or β1i may be useful to assess the role of each subunit in potentiating CFZ sensitivity and conferring resistance.

An important limitation to the study described in chapter 4 is the lack of distinction between the roles of the β1 and β1i subunits in conferring CFZ resistance. This is mainly due to a current lack of PIs that can selectively inhibit the activities of β1 and β1i. Our findings here provided initial clues to a potential role the β1 and β1i subunits may play in determining PI sensitivity. In order to further elucidate the mechanisms through which alterations in the β1 and/or β1i activities determine PI resistance, the most crucial next step is the development of PIs with better β1/1i selectivity. In general, little is known about how the different activities of the proteasome impact cell survival and apoptosis, and whether the unique activity of each catalytic subunit may have different roles in regulating specific cellular processes. In this regard, it would also be interesting to determine the substrates and pathways affected by selective inhibition of the β1 and β1i activities compared to inhibition of the CT-L activities. Furthermore, it would be important to further validate β1 upregulation as a resistance-conferring mechanism by determining whether transient or

stable overexpression of β1 may result in similar decreases in CFZ and BTZ sensitivities.

Chapter 5 addressed the poor metabolic stability of CFZ and its potential contribution to the intrinsic CFZ resistance against solid cancer from a drug delivery perspective. Our findings demonstrated that polymer micelle formulations of CFZ could protect the metabolic degradation of CFZ and prolong CFZ release *in vitro*. When tested in cells, these alternative CFZ formulations achieved equivalent or improved cell-killing activities against both lung cancer and MM cells. This was a proof-of-concept study to examine whether alternative CFZ formulations could potentially improve CFZ activity. Moving forward, it would be important to further assess the abilities of these polymer micelle formulations to protect CFZ from metabolic degradation *in vivo*. One of the major concerns with using a micelle-based formulation is its stability *in vivo*. Thus, a more careful characterization of particle stability *in vivo* is necessary to reveal whether these particles can remain intact and delivery the drug to a peripheral tumor site. Since our hypothesis was based on the assumption that metabolic instability and insufficient access of active CFZ to the tumor site was a major reason for the lack of anti-cancer activity against solid tumors, it would be important to compare the anti-cancer effects of CFZ entrapped in polymer micelles in *in vivo* models of solid cancer. To better understand how polymer micelles may contribute to a difference in anti-cancer activity, it would be necessary to not only assess the effect on tumor growth, but also compare the differences in biodistribution and proteasome inhibition between polymer micelle and the current clinical formulations of CFZ.

The studies described in this dissertation tackled the problem of CFZ resistance from three different perspectives of CFZ therapy. These findings contribute to our knowledge of CFZ resistance by providing initial evidence for novel mechanisms involved in determining CFZ sensitivity and novel strategies that can be utilized to combat resistance.

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311. Tsukioka, Y., et al., *Pharmaceutical and biomedical differences between micellar doxorubicin (NK911) and liposomal doxorubicin (Doxil).* Jpn J Cancer Res, 2002. **93**(10): p. 1145-53.
312. Li, L., et al., *Facile and Scalable Synthesis of Novel Spherical Au Nanocluster Assemblies@Polyacrylic Acid/Calcium Phosphate Nanoparticles for Dual-Modal Imaging-Guided Cancer Chemotherapy.* Small, 2015.
313. Perkin, K.K., et al., *Fabrication of hybrid nanocapsules by calcium phosphate mineralization of shell cross-linked polymer micelles and nanocages.* Nano Lett, 2005. **5**(7): p. 1457-61.
314. Oe, Y., et al., *Actively-targeted polyion complex micelles stabilized by cholesterol and disulfide cross-linking for systemic delivery of siRNA to solid tumors.* Biomaterials, 2014. **35**(27): p. 7887-95.
315. Vakil, R. and G.S. Kwon, *Effect of cholesterol on the release of amphotericin B from PEG-phospholipid micelles.* Mol Pharm, 2008. **5**(1): p. 98-104.

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EDUCATION

2007-2011 B.S. in Biochemistry
University of North Carolina at Chapel Hill
Chapel Hill, NC

PUBLICATIONS:

1. Ao L, Wu Y, Kim D, Jang ER, Kim K, Lee DM, Kim KB, Lee W. Development of Peptide-Based Reversing Agents for P-Glycoprotein-Mediated Resistance to Carfilzomib. Mol Pharm. 2012. 9 (8): 2197-205. PMC3473138
2. Ao L, Reichel D, Hu D, Jeong HY, Kim KB, Lee W, Bae YS. Polymer micelle formulations of proteasome inhibitor carfilzomib for improved metabolic stability and anti-cancer efficacy. J Pharmacol Exp Ther. 2015. 355 (2): 168-73. PMC4613964
3. Miller Z, Ao L, Kim, KB, Lee, W. Inhibitors of the Immunoproteasome: Current Status and Future Directions. Curr Pharm Des. 2013. 19(22): 4140-51. PMC3821965
4. Park J, Ao L, Miller Z, Kim K, Wu Y, Jang ER, Lee EY, Kim KB, Lee W. PSMB9 codon 60 polymorphism have no impact on the activity of the immunoproteasome catalytic subunit β1i expressed in multiple types of solid cancer. PLoS One. 2013. 8 (9): e73732 PMC3767749
5. Miller Z, Kim KS, Lee DM, Kasam V, Baek SE, Lee KH, Zhang YY, Ao L, Carmony K, Lee NR, Zhou S, Jeong HY, Zhan CG, Lee W, Kim DE, Kim KB. Proteasome inhibitors with pyrazole scaffolds from structure-based virtual screening. J Med Chem. 2015. 58 (4):2036-41. PMID25658656
6. Ao L, Lee MJ, Jang YJ, Lee DM, Lee W, Kim KB. Inhibition of the caspase-like proteasome activity restores sensitivity in carfilzomib-resistant cancer cells. *In preparation.*
7. Ao L, Lee W, Kim KB. Resistance mechanisms to proteasome inhibitor cancer therapy. *In preparation.*

Awards and Honors:

1. Kentucky Opportunity Fellowship (2013-2014): $15,000 plus a tuition scholarship and health insurance. University-wide competitive, awarded based on recommendation of the pharmaceutical sciences graduate program indicating high confidence in one's academic potential
2. American Foundation for Pharmaceutical Education (AFPE) Fellowship (2014-2016): $6500 toward stipend for 2014-2015, renewed for 2015-2016. Nationally competitive pre-doctoral fellowship awarded to 50 exceptional students each year to support their efforts in pharmaceutical-oriented areas of research.
3. University of Kentucky Dissertation Year Fellowship (2015-2016): $20,000 plus tuition scholarship and health insurance. University-wide competitive, awarded based on recommendation of the department and graduate school selection committee. Indicates high confidence in one's academic potential.
4. 1st place poster presentation award at 2015 Markey Cancer Center Research Day

LEADERSHIP EXPERIENCE:

2012-2015	Chair of the Pharmaceutics Graduate Student Research Meeting (PGSRM)
2013-2014	Chair of the American Association of Pharmaceutical Scientists Student Chapter
2013-2014	Graduate Student Representative of the Drug Discovery and Develop Symposium (2014)
2012-2013	Treasurer of the American Association of Pharmaceutical Scientists Student Chapter (2012-13)

ORAL PRESENTATIONS:

1. Ao L, Wu Y, Lee DM, Kim KB, Lee W. "Mechanisms of resistance to proteasome inhibitor chemotherapy". UK Pharmaceutical Sciences Drug Discovery Seminar, Lexington KY, May 10, 2013.
2. Ao L, Hu D, Jeong HY, Kim KB, Lee W. "Investigation of mechanisms underlying acquired resistance to carfilzomib in human pancreatic cancer cells" Pharmaceutics Graduate Student Research Meeting, Chicago, IL, June 27, 2014.

3. Ao L, Reichel D, Hu D, Jeong HY, Kim KB, Bae Y, Lee W. "Polymeric micelle formulations to improve anti-cancer efficacy of carfilzomib". UK Pharmaceutical Sciences Student Seminar Series, Lexington KY, September 12, 2014.
4. Ao, L, Lee MJ, Lee DM, Jang YJ, Lee W, Kim KB. "Inhibition of C-L Proteasome Activity Restores CFZ Sensitivity in Pancreatic Cancer and Multiple Myeloma Cells". Workshop for Japan-Korea Young Scientists in Pharmaceutics. Kyoto, Japan, June 25, 2016.

POSTER PRESENTATIONS (SELECT):

1. Ao L, Wu Y, Kim D, Jang ER, Kim K, Lee DM, Kim KB, Lee W. "Development of Peptide-Based Reversing Agents for P-Glycoprotein-Mediated Resistance to Carfilzomib". Pharmaceutics Graduate Student Research Meeting, Omaha NE, June 8, 2012; and Annual Symposium on Drug Discovery and Development, Lexington KY, September 20, 2012.
2. Ao L, Kim KB, Park J, Li YJ, Lee, W. "Identification and functional characterization of novel splicing variants for the immunoproteasome catalytic subunits β1i and β2i". American Association of Cancer Research Annual Meeting, Washington, D.C., April 9, 2013; and Pharmaceutics Graduate Student Research Meeting, Iowa City, Iowa, June 7, 2013.
3. Ao L, Park J, Hu D, Jeong HY, Kim KB, Lee W. "Accelerated proteasome turnover as a potential mechanism of acquired resistance to carfilzomib in BxPC3 pancreatic cancer cells". American Association of Cancer Research Annual Meeting, San Diego, CA, April 5, 2014.
4. Ao L, Reichel D, Hu D, Jeong HY, Kim KB, Bae Y, and Lee W. "Nanoparticle formulations of carfilzomib for improved metabolic stability and anti-cancer efficacy". Experimental Biology Annual Meeting, Boston, MA, March 29, 2015; Markey Cancer Center Research Day, Lexington, KY, May 28, 2015. Winner of 1st Place in Poster Presentation Awards
5. Ao L, Carmony KC, Lee MJ, Park CW, Lee W, and Kim KB. "Alterations in cellular proteasome catalytic subunit composition as a potential mechanism of acquired resistance to proteasome inhibitor therapy". Discovery on Target, Boston, MA, September 23, 2015.
6. Ao L, Lee W, Kim KB. "Tyrosine kinase inhibitors potentiate anti-myeloma activity of proteasome inhibitor carfilzomib in resistant multiple myeloma cell lines". International

Society for the Study of Xenobiotics (ISSX) Meeting, Busan, South Korea, June 12-16, 2016.

UNDERGRADUATE RESEARCH:

University of Kentucky Superfund Summer Research Program (May-August, 2010)

Project: Mechanisms of inflammatory responses and degradation of blood brain barrier as a consequence of exposure to polychlorinated biphenyls

Mentor: Dr. Michal Toborek (Department of Neurosurgery, College of Medicine)

University of North Carolina at Chapel Hill (January-May, 2010)

Project: P-glycoprotein-mediated drug resistance in breast cancer cells

Mentor: Dr. Kenneth Bastow (University of North Carolina Eshelman School of Pharmacy)

TEACHING & MENTORING:

1. Graduate Student Mentor for the Summer Undergraduate Research Program at University of Kentucky College of Pharmacy (2011-2015)
2. Teaching Assistant at the University of Kentucky College of Pharmacy (2011-2013)
3. Teaching Assistant at University of North Carolina-Chapel Hill Department of Chemistry (2011)

AFFILIATIONS:

American Association of Cancer Research (2012-2014)

American Association of Pharmaceutical Scientists (2011-2015)

American Association for the Advancement of Science (2012-present)

American Foundation for Pharmaceutical Education Fellow (2014-2016)