

## Review Article

# Potential usage of proteasome inhibitor bortezomib (Velcade, PS-341) in the treatment of metastatic melanoma: basic and clinical aspects

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**Abstract:** Protein degradation by proteasome is essential to the regulation of important cellular functions including cell cycle progression, proliferation, differentiation and apoptosis. Abnormal proteasomal degradation of key regulatory proteins perturbs the normal dynamics of these cellular processes culminating in uncontrolled cell cycle progression and decreased apoptosis leading to the characteristic cancer cell phenotype. Proteasome inhibitors are a novel group of therapeutic agents designed to oppose the increased proteasomal degradation observed in various cancers while restoring key cellular functions such as apoptosis, cell cycle progression, and the inhibition of angiogenesis. Several proteasome inhibitors have been evaluated in pre- and clinical studies for their potential usage in clinical oncology. Bortezomib (Velcade, PS-341) is the first Food and Drug Administration-approved proteasome inhibitor for the treatment of multiple myeloma and mantle cell lymphoma. Bortezomib's ability to preferentially induce toxicity and cell death in tumor cells while rendering healthy cells unaffected makes it a powerful therapeutic agent and has extended its use in other types of malignancies. The ability of bortezomib and other proteasome inhibitors to synergize with conventional therapies in killing tumors in various *in vitro* and *in vivo* models makes this class of drugs a powerful tool in overcoming acquired and inherent resistance observed in many cancers. This is achieved through modulation of aberrant cellular survival signal transduction pathways and their downstream anti-apoptotic gene products. This review will discuss the anti-neoplastic effects of various proteasome inhibitors in a variety of cancers with a special emphasis on bortezomib, its mechanism of action and role in cancer therapy. We further discuss the potential use of bortezomib in the treatment of metastatic melanoma.

**Keywords:** Melanoma, proteasome, apoptosis, immunoresistance, cell signaling, targeted therapy, NF- $\kappa$ B, bortezomib

## Introduction

The systematic and timely degradation of proteins is a vital process for cell function and maintenance of cellular homeostasis. It is for this reason that eukaryotic cells have evolved two different peptide degradation mechanisms: the ubiquitin-proteasome pathway and the lysosomal degradation pathway. The lysosomal degradation mechanism is responsible for both exogenous and endogenous peptide hydrolysis. In contrast to the ubiquitin-proteasome pathway, the lysosomal pathway is less specific in the proteins it degrades and leads to the destruction of both membrane-bound peptides and exogenous peptides engulfed through phagocytosis or endocytosis [1]. Dysfunctional cellular

organelles and endogenous proteins are also cleared by lysosomes, a process known as autophagy, allowing maintenance of cellular homeostasis and proper cell function [2]. However, the majority of endogenous proteins are degraded by the 26S proteasome [1].

The 26S proteasome plays a vital role in eukaryotic cell function and viability. It is responsible for a plethora of integral cellular processes including timely degradation of cell cycle regulator proteins, transcription factors and maintenance of cellular homeostasis, all of which are essential for cell proliferation, differentiation, angiogenesis, and apoptosis [3-5]. Cellular proteins destined for proteasome degradation are shuttled through a three-enzyme

pathway that adds multiple ubiquitin molecules to the protein. The poly-ubiquitination of peptides is intended to mark proteins for degradation and target them to the 26S proteasome [3]. Once specific marked peptides have entered the proteasome degradation ensues.

Ubiquitination of peptides begins with enzyme E1 activating the ubiquitin protein through adenylation of the C-terminal glycine followed by the formation of a thioester bond between the activated ubiquitin and E1. E2 then undergoes a trans-thioesterification allowing conjugation of the activated ubiquitin to E2. E3 recruits the substrate (protein destined for degradation) and transfers the activated ubiquitin to the peptide. The cycle then repeats, creating a poly-ubiquitinated substrate ready for recognition and degradation by the 26S proteasome [3,6]. The 26S proteasome is comprised of two parts: the 19S regulatory core and the 20S catalytic core. The regulatory core is responsible for recognition of the poly-ubiquitinated substrates and the shuttling of the substrate into the 20S catalytic core. The catalytic core then degrades the peptides through trypsin-, chemotrypsin- and caspase-like activity [3,7].

The proteasome has an important role in the control of regulated cell death, or apoptosis. There are two pathways that induce apoptosis: the intrinsic and the extrinsic pathways. The intrinsic and extrinsic pathways work in caspase-independent and caspase-dependent fashions, respectively; however, a particular family of proteins, the Bcl-2 family, has a role in regulating both pathways. The Bcl-2 family contains about 25 pro- and anti-apoptotic proteins that exist in a balanced ratio. The cell will undergo apoptosis when this ratio is disturbed in favor of the pro-apoptotic proteins, thus making this family of proteins an important target in cancer therapy [8]. The proteasome has been found to regulate the levels of the Bcl-2 family [9] as well as other mediators of apoptosis through direct or indirect modulation [10], signifying its importance in apoptosis.

The proteasome does not solely destroy proteins, but can also modify protein length and, thus, change protein function considerably. Among these substrates are cell cycle regulators, tumor suppressors and transcription factors. In terms of apoptosis, nuclear factor kappa B (NF- $\kappa$ B) is a family of dimeric transcription

factors that have been implicated in cell survival [11]. The NF- $\kappa$ B family is controlled by a family of inhibitory proteins, I $\kappa$ B, that bind to NF- $\kappa$ B and prevent nuclear translocation. Proteasomal degradation of I $\kappa$ B permits the activation and translocation of NF- $\kappa$ B to the nucleus to initiate transcription of important survival factors that prevent apoptosis [12]. Hyperactivity of the NF- $\kappa$ B pathway is a hallmark of many cancers, including melanoma and multiple myeloma (MM). This aberrant activity promotes cancer development through expression of, among other things, cell cycle genes, chemoresistance and apoptotic inhibitors [13-15].

As mentioned, the 26S proteasome of eukaryotic cells plays a crucial role in the regulation of various cellular processes through protein degradation. Of these processes cell proliferation, cell survival signaling cascades and cell differentiation are fundamental in promoting the evasive phenotype of tumor cells. Therefore, the discovery of proteasome inhibitors provides a novel approach in selectively inducing apoptosis, reducing cell proliferation and sensitizing tumor cells to cytotoxic T-cell (CTL) attack as well as conventional therapeutics. In this review we will briefly discuss the roles of various proteasome inhibitors in a range of cancers, with an emphasis on bortezomib (Velcade, PS-341) and its potential use in melanoma treatment.

### Proteasome inhibitors

The cancer cell phenotype is characterized by abnormal cell proliferation, resistance to apoptosis, increased angiogenesis and resistance to CTL killing. This lack of susceptibility to CTL lysis while also exhibiting an increase in the expression of survival proteins, poses a significant challenge in developing effective cancer therapies. Knowing that cancer cells are highly dependent on the proteasome [16], have enhanced proteasomal activity [4] and are more responsive to the effects of proteasome inhibition [17], proteasome inhibitors pose as a novel clan of anti-cancer therapeutics.

Anti-cancer drugs and therapies are extremely challenging to develop and are both time and cost extensive procedures. However, there is a growing need in finding effective therapies to combat malignancies. Due to the proteasome's ubiquitous presence in cellular processes, pro-

teasome inhibitors potentially pose as a novel therapeutic against cancer. Proteasome inhibitors have been known to induce apoptosis and toxicity specifically in cancer cells while rendering normal cells unaffected [18-20]. Several proposals have been used to describe the mechanism in which proteasome inhibitors induce apoptosis. Mechanisms, among others, include the up-regulation of NOXA [21], an increase in pro-apoptotic Bcl-2 proteins [8] and inhibition of the NF- $\kappa$ B pathway [22].

Proteasome inhibitors come in two different types: synthetic and natural inhibitors. Synthetic inhibitors are usually composed of a peptide backbone attached to a "warhead" that disrupts the proteasome's degradative abilities. These compounds mimic proteasome substrates and bind into the proteasome's active site disrupting its degradation capability [23]. Natural products, which are not peptide-based, such as polyphenol (-)-epigallocatechin-3-gallate (EGCG), soy isoflavonoids and the spice curcumin, have shown efficacy in treating various cancers, both in combination with traditional chemotherapeutic drugs and when used alone [24]. Lactacystin and salinosporamide A are additional examples of natural proteasome inhibitors [23].

Natural inhibitors are found throughout everyday environments. One of which is Lactacystin, which was the first natural proteasome inhibitor discovered in *Streptomyces* [25]. Unlike other proteasome inhibitors such as bortezomib and MG132, lactacystin inhibits the proteasome through non-reversible covalent bonds at the N-terminus threonine residue in the  $\beta$ -1 subunit of the proteasome [26]. There is evidence that lactacystin induces apoptosis in prostate cells, which coincided with a substantial decrease in NF- $\kappa$ B activity and decrease in pro-survival proteins. In combination with MG132, lactacystin induced apoptosis 39% more effectively. Enhanced apoptosis coincided with concomitant down-regulation of pro-survival proteins, such as Bcl-2 and Mcl-1 [26].

(-)-EGCG, a green tea polyphenol, has been shown to effectively and selectively inhibit the proteasome in breast tumors [27], hepatocellular carcinoma HepG2 and cervical carcinoma HeLa cells [28]. *in vivo* studies have shown (-)-EGCG to decrease breast cancer growth and induce apoptosis [27]. (-)-EGCG has also been

shown to selectively inhibit tumor cell survival pathways and cell proliferation [29]. Its mechanism of action involves selective disruption of the chymotrypsin-like activity of the proteasome [28].

Another natural proteasome inhibitor comes from the spice curcumin. After treating oesophageal cancer cells in a 24h study with 5-50  $\mu$ M doses of curcumin, non-apoptotic cell death was observed. Further examination revealed an accumulation of poly-ubiquitinated proteins and cyclin B, hallmarks of proteasomal dysfunction. This inhibition could be indicative of the non-viability of these oesophageal cancer cells [30]. Also, in human prostate cancer cells, the natural proteasome inhibitor NPI-0052 (salinosporamide A) is also a potent anti-cancer therapeutic. NPI-0052 inactivates the NF- $\kappa$ B pathway and modulates the metastasis inducer Snail and suppressor Raf-1 kinase inhibitor protein (RKIP). Inhibition of this regulatory circuit leads to down-regulation of anti-apoptotic products, specifically reduction in Snail expression and induction of RKIP, which leads to sensitization to cisplatin (CDDP) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [31].

TRAIL is a potent inducer of the extrinsic apoptosis cascade, a function that is often suppressed in cancer cell lines. Colon carcinoma cells treated with recombinant human TRAIL (rhTRAIL) did not undergo apoptosis. By pre-treatment with MG132 the acquired TRAIL-resistance was reversed [32]. MG132 is a peptidyl aldehyde whose mechanism of proteasome inhibition is through reversible binding to the N-terminus threonine in the  $\beta$ -1 subunit of the 26S proteasome [26]. Resistance was a result of a decreased pro-caspase-8/cFLIP (caspase-8 inhibitor) ratio. After proteasome inhibition by MG132, caspase-8 protein levels were stabilized, thus, reversing resistance [32].

One surprising proteasome inhibitor that has shown unexpected, yet potent activity is disulfiram [14]. Disulfiram was originally shown to be an inhibitor of the enzyme alcohol dehydrogenase leading to its widely used in the treatment of alcoholism for the past fifty years. New studies have shown that disulfiram can be used as an anti-tumor and chemosensitizing therapeutic [33,34]. Disulfiram forms a complex with copper leading to proteasome inhibition through

disruption of its chymotryptic-like activity and inducing apoptosis [33,34]. Highly elevated serum levels of copper have been observed in patients with breast, prostate and brain cancer, allowing disulfiram to be a highly selective proteasome inhibitor in these settings. Furthermore, it has been indicated that copper is a vital constituent in tumor angiogenesis [35,36]. These results implement disulfiram as a promising candidate for cancer treatment inducing selective toxicity restricted to tumor cells.

Much like other proteasome inhibitors, carfilzomib, an epoxomicin-related inhibitor, blocks the chymotryptic-like activity of the proteasome [37]. Carfilzomib substantially suppresses tumor cell proliferation and progression. This leads to eventual apoptosis through various mechanisms including the activation of c-Jun-N-terminal kinase (JNK-1), which is involved in cytochrome c release, mitochondrial membrane depolarization, activation of both caspase pathways and apoptosis. Furthermore, carfilzomib has shown to be highly potent in MM patient cells and cell lines that are normally resistant to bortezomib treatment [37].

Lastly, four structurally different proteasome inhibitors, Acetyl-leu-leu-norleu-al (ALLN), MG132, epoxomicin, and bortezomib, each effectively inhibited the *in vitro* growth of melanoma cells as evidenced by reduction of the cell proliferation rate and through induction of caspase-dependent and -independent cell death [38]. This study suggests the potential efficacy of proteasome inhibitors as important cancer therapeutic agents against melanoma and many other cancers, and merits further evaluation.

### **Bortezomib (Velcade, PS-341)**

Bortezomib is a dipeptide boronic acid analog [39,40] that shows extreme selectivity of action towards cancer cells' proteasome, giving it a distinct advantage as a therapeutic agent [41]. Its mode of inhibition is through reversible binding to the N-terminus threonine residue in the  $\beta$ -1 subunit of the catalytic core complex of the 26S proteasome [26,42], leading to reversible inhibition of the chymotrypsin-like and proteolytic activity of the proteasome [43]. This results in several biological effects, including inhibition of the cell cycle, increased apoptosis, inhibition of NF- $\kappa$ B activity, induction of ER stress and sensitization of the tumor cells to

drugs and CTL lysis [44].

Bortezomib is the first Food and Drug Administration (FDA)-approved proteasome inhibitor for cancer treatment [45,46]. In 2003, the FDA granted accelerated approval for the marketing of bortezomib (Velcade, PS-341) as a single agent for the treatment of MM. Approval was based on phase II clinical trials where of the 188 patients enrolled with MM, 52 (27.7%) experienced either a complete remission (CR) or partial response (PR) with bortezomib treatment. The recommended dosage was 1.3mg/m<sup>2</sup> and was approved for usage in patients who had previously received at least two prior treatments and have continued disease progression during their last treatment [43]. Later in 2005 the FDA granted the use of bortezomib in patients with only one prior treatment [47]. Then in 2006, the FDA granted the use of bortezomib in patients with Mantle Cell Lymphoma (MCL) who have underwent one prior treatment [48].

### **Bortezomib in cancer therapy**

Aside from its well established clinical efficacy in patients with MM [43,47] and MCL [48], bortezomib recently has been used in a plethora of other cancers including: adult T-cell leukemia/lymphoma (ATLL), lung cancer, breast cancer, prostate cancer, pancreatic cancer, head and neck cancer, melanoma and colon cancer [49], neuroblastoma [50], and cutaneous T-cell lymphoma (CTCL) [51].

In MM, bortezomib activates tumor suppressor genes and increases the expression of pro-apoptotic proteins Bid, Bax, and caveolin-1, while inhibiting NF- $\kappa$ B [49]. Bortezomib treatment resulted in decreased tumor growth, angiogenesis, metastasis and increased apoptosis. In pancreatic cells treated with bortezomib considerable repression in Bcl-2 and an increase in Bax and p53 was observed. This resulted in diminished cancer cell proliferation and augmented apoptosis. The level of apoptosis significantly increased when bortezomib was used in combination with gemcitabine [52].

Proteasomal inhibition has been shown to be effective in restoring the TRAIL-mediated apoptotic signaling pathway. Esophageal squamous cell carcinomas (ESCC) treated with bortezomib and TRAIL showed enhanced susceptibility to TRAIL-induced apoptosis as well as increased association of caspase-8 and the Fas-

associated death domain (FADD) to the death-inducing signaling complex (DISC) [44]. The mechanisms by which sensitivity was induced differed among individual ESCC lines, but the processes included both extrinsic and intrinsic apoptotic pathways where amplified caspase-8 activation along with c-FLIP inhibition and increased expression of caspase-9 was observed, respectively. Similarly, certain human renal cell carcinoma (RCC) lines were sensitized to TRAIL upon bortezomib treatment. Sensitization was not due to an up-regulation of TRAIL receptors (DR4, DR5) or down-regulation of Bcl-2 and IAP family members, but rather through an increase in caspase-8 activity [53]. Thus, bortezomib uses distinct mechanisms to sensitize tumors to TRAIL.

Bortezomib induces apoptosis in CTCL and ATLL via down-regulation of anti-apoptotic factors c-Flip and X-linked inhibitor of apoptosis (XIAP) most likely caused by the inactivation of the NF- $\kappa$ B pathway. Furthermore, up-regulation of NOXA, a pro-apoptotic factor of the BH3-only family, was shown in both transcript and protein levels. NOXA co-precipitates with anti-apoptotic Mcl-1, implying that this interaction is a vital component for bortezomib-induced apoptosis in CTCL and ATLL [51].

Constitutive hyperactivity of the NF- $\kappa$ B pathways confers a survival advantage to tumors and endows resistance to apoptosis [54]. Increased activity of the NF- $\kappa$ B pathway has been observed in various tumor types including breast, colon, prostate [55] and melanoma [15,56,57] and is therefore an attractive target for cancer therapeutics. In colorectal and carcinoma cell lines proteasome inhibition by bortezomib interferes with NF- $\kappa$ B signaling and prevents its translocation into the nucleus. Thus, the activation of downstream anti-apoptotic agents does not occur [58]. Moreover, B-cell lymphoma lines perturbed by heat shock and then treated with bortezomib undergo apoptosis, as evidenced by inhibited NF- $\kappa$ B activation, up-regulated caspase-3 activity and down-regulated anti-apoptotic protein, inhibitor of apoptosis protein (cIAP-2) [54]. Bortezomib also acts synergistically with histone deacetylase inhibitors (HDACis) to induce apoptosis through inhibition of NF- $\kappa$ B by down-regulating NF- $\kappa$ B target genes, such as c-MYC and IKK [59]. Although inhibition of NF- $\kappa$ B is a promising therapy for some cancers, it has been shown to be ineffective in others [60], requiring that other

mechanisms of inducing apoptosis be elucidated for other potential therapies.

An emerging mechanism employed by bortezomib to induce apoptosis is the creation of ER stress through the accumulation of misfolded proteins inside the cell. These proteins induce homeostatic repair pathways that lead to programmed cell death [61]. Insight into this mechanism was revealed when a point mutation found in the proteasome  $\beta$ -5 subunit (PSMB5) in MM cell lines was found to contribute to resistance against bortezomib-induced apoptosis. This mutation prevented the accumulation of misfolded, poly-ubiquitinated proteins that trigger ER stress [62]. Neuroblastoma cells treated with bortezomib induced eukaryotic initiation factor (eIF2 $\alpha$ ) signaling which is correlated with increased NOXA expression as well as increased phosphorylation of activating transcription factor-3 (ATF3), ATF4, growth arrest and DNA damage-inducible protein (GADD34). All of these proteins are associated with ER stress [50]. In diffuse lymphocytic B-cell lymphoma cells treated with bortezomib and HA14-1 (Bcl-2 antagonist) an increase in Bax, Bak, cytochrome c release, caspase activation, as well as increased activation of c-Jun N-terminal Kinases (JNK) was noticed, resulting in ER stress and later apoptosis [63].

Bortezomib has selective cytotoxicity towards hypoxic tumor cells than to normoxic. Treatment of hypoxic cells with cyclohexamide, which relieves the ER load, altered the enhanced cytotoxicity of bortezomib. This indicates that ER stress is indicative in the increased cytotoxicity of bortezomib in hypoxic tumor cells [64]. Cellular proliferation and progression in many human cancers is regulated by the epidermal growth factor receptor (EGFR), which is ubiquitinated and degraded by the proteasome [65]. ER stress can be induced in EGFR inhibitor-resistant cancer cells treated with bortezomib leading to apoptosis from the resulting increased cleavage of pro-apoptotic protein Bid and caspase-8 activation [65]. It is apparent that the induction of ER stress-induced apoptosis by bortezomib is an effective method in promoting selective apoptosis. Determining the precise mechanism employed by bortezomib is a great step toward achieving more effective cancer therapies in the future.

Previously, it has been reported that bortezomib has a role in sensitizing tumor cells to CTL kill-

ing [66,67]. Human papillomavirus type 16 (HPV-16) is associated with close to 50% of cervical cancers and, although an attractive target for CTL-mediated immunotherapy, there have been only a few CTL epitopes that have been associated with HPV-16 [41]. A CTL clone generated against the E6 epitope was unable to recognize the MHC-complex unless the cancer cells were pre-treated with bortezomib and interferon- $\gamma$  (IFN- $\gamma$ ). The cells then became susceptible to CTL lysis due to bortezomib's ability to increase recognition between CTLs and the MHC complex [41]. Similarly, mice vaccinated with an HPV-16 E7 antigen and then treated with bortezomib generated more potent E7-specific CD8 $^{+}$  T cell immune responses against the tumor cells compared to monotherapy results [68]. Bortezomib treatment led to increased apoptosis in the tumor cells and caused them to be more susceptible to lysis by E7-specific CTLs, which may be due to an up-regulation of MHC class I, due to an increased expression of E7 protein, or death receptors [68]. Although some cancer cells can be sensitized to CTL lysis by bortezomib, not all behave with similar effects and the mechanism employed may function differently in other forms of cancers. For example, augmenting caspase-8 activity by bortezomib also renders tumors susceptible to natural killer (NK) cell lysis, but alternatively renders the cells resistant to CTL killing [69]. Therefore, it is critical to develop an in depth understanding of the different mechanisms of action of bortezomib in order to utilize it as a strong cancer therapeutic.

### Bortezomib in melanoma therapy

Metastatic melanoma is one of the most biologically aggressive and notoriously chemoresistant cancers known. Melanoma occurs as a result of genetic and/or epigenetic events that activate various oncogenes that give the altered melanocytes a growth advantage over normal melanocytes. Many of these genetic changes alter pathways involved with cell proliferation and survival, which play a major role in forming a tumor cell phenotype. However, the most important phenotypic change is the reduction of apoptosis through upregulation of anti-apoptotic gene products [70]. Overall, these anti-apoptotic genes are up-regulated due to hyperactivity of the NF- $\kappa$ B pathway, a hallmark of melanoma [15,56,57]. Current studies reveal that treatment with bortezomib in melanoma has led to

reversal of CTL resistance [71], a decrease in cellular growth and an increase in apoptosis, although more significant results occur when bortezomib is used in combination with other therapies.

Tumor cells previously resistant to CTL attack can be sensitized by bortezomib treatment. Established melanoma lines and primary melanoma lines notoriously resistant to CTL attack were sensitized to melanoma reactive CTLs after treatment with bortezomib. The underlying mechanism for this enhanced sensitivity was due to an increased induction of NOXA. This up-regulation of NOXA induces the mitochondria to release second mitochondria-derived activator of caspase (SMAC), a pro-apoptotic protein that regulates the intrinsic apoptotic pathway, leading to enhanced caspase activation and mediation of CTL lysis [72]. This suggests that bortezomib does not alter the surface expression of melanoma specific antigens recognized by CTLs. c-MYC, an oncogene that is regulated by the proteasome, is highly up-regulated during tumor progression. c-MYC down-regulation is associated with low levels of pro-apoptotic NOXA. Interestingly, artificial up-regulation of c-MYC leads to NOXA production resulting in cell death. This indicates the involvement of an oncogenic pathway, which may confer sensitivity to proteasome inhibition [20]. Bortezomib treatment sensitizes the B16 murine melanoma model to dendritic cell (DC)-activated effector cells, including CD8 $^{+}$  cells and NK cells. This enhanced sensitivity is mediated by TNF- $\alpha$  and NF- $\kappa$ B inhibition [73].

Nevertheless, bortezomib, as a single agent monotherapy, is not sufficient to induce a strong response to lysis by CTLs. However, in combination with various other treatments the efficiency of bortezomib is substantially increased, as is seen in melanoma cells treated with bortezomib and temozolomide, a standard chemotherapeutic. This combination inhibits melanoma growth in a murine model, resulting in CR after 30 days of therapy that lasted more than 200 days [15]. Similar effects were seen when bortezomib treatment was combined with rosiglitazone [74].

Increased levels of XIAP are found in many cancers including melanoma. It significantly impacts the apoptosis threshold through its ability to disrupt and block cell caspase activation.

XIAP-knockdown, in combination with bortezomib, resulted in a substantial increase of ER stress-induced apoptosis of melanoma cell lines, identifying XIAP as a potential target for melanoma therapy [75]. SMAC is known to antagonize XIAP and potentially reverses chemoresistance in tumors. Recently, a study using SMAC-mimetics and bortezomib effectively induced apoptosis in melanoma cell lines. This combination also overcame melanoma resistance to the combination of SMAC-mimetics and TRAIL [76]. A study employing the combination of camptothecin and bortezomib resulted in a drastic increase in the anti-tumoral effects of camptothecin. This combination showed drastic changes in tumor growth and reduced pulmonary melanoma metastasis compared to each agent used individually [77]. Many melanoma lines have high levels of Bcl-2 family proteins, which contributes to the resistance seen in melanoma lines [71]. Melanoma cells treated with bortezomib and (-)-gossypol, Bcl-2 family inhibitor, showed more effective induction of apoptosis *in vivo* [78]. Furthermore, Bortezomib and IFN- $\alpha$  act synergistically to overcome Mcl-1 and Bcl-2 overexpression [79].

Although combination therapies with bortezomib seem to be more effective in cancer treatment, there has been evidence that, at times, combination strategies are less effective. Using B16F10 melanoma cell lines, bortezomib was given both as a single agent and in combination with the heat shock protein (Hsp) 70 inhibitor, quercetin. In cell lines treated only with bortezomib, cells shrunk and detached. Interestingly, neither the combination of bortezomib and quercetin nor quercetin alone produced the morphological changes as seen with bortezomib treatment. These results indicate that quercetin antagonizes bortezomib's anti-neoplastic effects rather than improving its efficacy in melanoma treatment [80]. Clearly, bortezomib works through multiple mechanisms to achieve cell death and does not necessarily act consistently among various cancers. Thus, the development of a battery of therapies, both combinatorial and single-agent strategies, may be necessary to successfully treat melanoma and other cancers.

Although bortezomib down-regulates anti-apoptotic proteins such as Bcl-2 [80], Mcl-1 [79,81] and XIAP [75], it also potentiates pro-apoptotic proteins to help mediate its effects.

The simultaneous up-regulation of NOXA, with bortezomib treatment, and the down-regulation of Mcl-1, with small interfering RNA (siRNA), enhances melanoma killing [81]. When NOXA was disrupted through RNA interference (RNAi) apoptosis was reduced by 30-50% in melanomas [82]. NOXA up-regulation was also found to occur in both *in vitro* and *in vivo* studies after bortezomib treatment [83]. Furthermore, another study using a genome wide siRNA of three cancer cell lines, including melanoma, identified 39 proteins important in bortezomib-induced cell death, one of which was NOXA [84]. Bim, another pro-apoptotic protein, has been singled out as a target for proteasome degradation. Treatment with bortezomib leads to the induction of NOXA production causing the dissociation of Bim from Mcl-1. This causes activation of other pro-apoptotic proteins eventually leading to cell death [85]. In murine B16 melanoma cells, TGF- $\beta$  inducible early gene (TIEG1) was significantly up-regulated by bortezomib, as were the levels of Bax and Bim. The levels of cytochrome c and caspase-3 activation also increased due to mitochondrial collapse associated with intrinsic apoptotic pathway [86]. Again using murine B16 melanoma cells, bortezomib treatment inhibited NF- $\kappa$ B and significantly reduced tumor size. Furthermore, combination treatment with temozolomide induced CR in mice which lasted more than 200 days [15].

As previously mentioned, bortezomib is not always sufficient to induce apoptosis in melanoma cells as it can ineffectively down-regulate Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 and at times even up-regulate anti-apoptotic factors [81]. Bortezomib in combination with INF- $\alpha$  [79], IL-29 [87], dexamethasone [88] or fenretinide [89], resulted in increased melanoma cell death compared to monotherapy with bortezomib. Bortezomib, although a very promising cancer therapeutic, clearly works most effectively in combination with other therapeutic agents.

### Conclusion

Proteasome inhibition is a distinctive and novel therapy against many cancers and causes a reversal in cancer phenotypes including an increase in apoptosis, decrease in cellular growth, and sensitization to CTL lysis. Bortezomib has been the first and most widely used proteasome inhibitor but its efficacy is limited when used as a single agent. However, combined with other

therapeutic agent, its efficacy increases. Clinical studies attempting to determine the effect of bortezomib against melanoma have not yet observed a major response to bortezomib in patients, as one study found only 22% of patients that achieved stable disease [90] when treated with bortezomib, indicating a need to discover the most effective dose while also limiting toxicities in patients. Toxicities such as diarrhea, fatigue and thrombocytopenia have been observed in lymphoma patients treated with bortezomib [91] while side effects of erythematous plaques, purpuric eruptions, folliculitis, Sweet's syndrome and leukocytoclastic vasculitis have been observed in dermatologic diseases treated with bortezomib [92]. Bortezomib resistance in tumors has also been observed as an emerging challenge to cancer therapy [42,93], which makes understanding the precise mechanism of bortezomib vital. Furthermore, the discovery of other molecular participants in its inhibitory pathway, while combining other anti-cancer treatments and focusing on the development of more effective proteasome inhibitors, is an essential step to the successful treatment of cancer.

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