

# Chapter 1

## Proteasome Inhibitors and Lessons Learned from Their Mechanisms of Action and Resistance in Human Cancer

Sara M. Schmitt, Rahul R. Deshmukh, and Q. Ping Dou

**Abstract** Selective protein degradation by the ubiquitin–proteasome pathway (UPP) is critical to cellular homeostasis, and dysregulation of the UPP has been associated with human diseases including cancer. Proteasome inhibition as a strategy for cancer treatment was validated by the US Food and Drug Administration approval of the proteasome inhibitor bortezomib for the treatment of multiple myeloma in 2003. After 10 years of success, bortezomib and its combinational therapies have become a staple for treating relapsed/refractory multiple myeloma. Unfortunately, bortezomib has several limitations, including, most notably, the emergence of resistance. To overcome bortezomib resistance, several approaches have been taken, including the development of novel second-generation proteasome inhibitors, application of rationalized bortezomib-based combinational therapies, and targeting sites outside the proteasomal core as well as factors involved in resistance mechanisms. Further understanding the mechanisms of resistance to proteasome inhibitors in human cancers will significantly improve current proteasome inhibitor therapies and patient care.

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## Abbreviations

BMSC	Bone marrow stromal cell
CQ	Clioquinol
CT	Chymotrypsin
DSF	Disulfiram
DUB	Deubiquitinating enzyme
(-)-EGCG	(-)-Epigallocatechin-3-gallate
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
HDAC	Histone deacetylase
HSP	Heat shock protein
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
IPSI	Immunoproteasome-specific inhibitor
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
NSCLC	Non-small cell lung cancer
PBMC	Peripheral blood mononuclear cell
PGPH	Peptidyl-glutamyl peptide-hydrolyzing
RIP-1	Regulatory particle inhibitor peptoid-1
ROS	Reactive oxygen species
SAR	Structure–activity relationship
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
UIM	Ubiquitin-interacting motif
UPP	Ubiquitin–proteasome pathway

## 1.1 Introduction

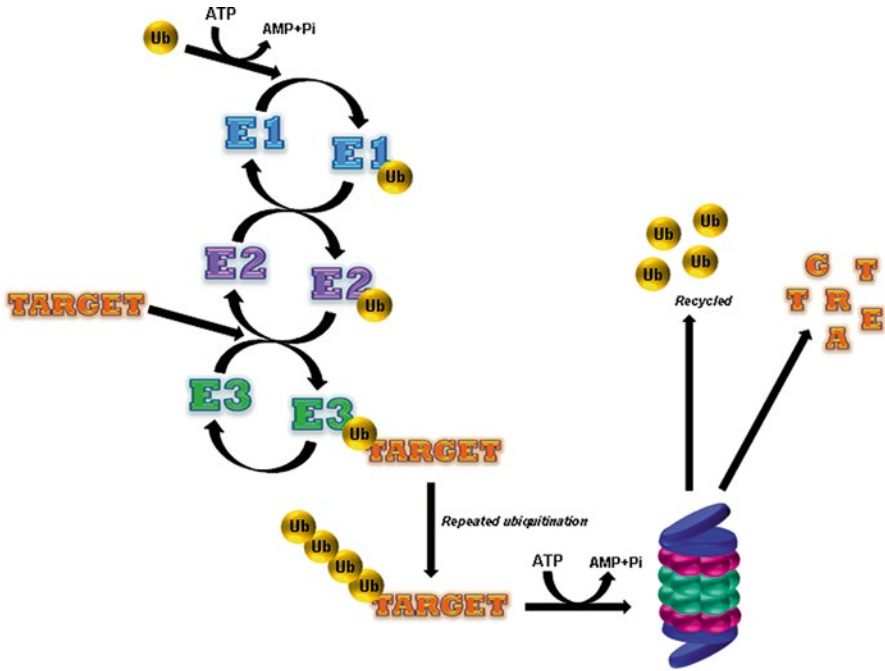
The ubiquitin–proteasome pathway (UPP) has gained considerable attention as a potential target for cancer therapeutics, owing to its extreme importance to normal cellular function and dysregulation in malignant cells. In fact, critical proteasomal target proteins are involved in processes important for carcinogenesis, including cell cycle progression, proliferation, and differentiation. The past decade has witnessed the emergence of proteasome inhibition as an effective therapeutic strategy for treating multiple myeloma. Bortezomib was the first proteasome inhibitor approved by the US Food and Drug Administration (FDA) in 2003, and the use of bortezomib

and bortezomib-based combinational therapies has become a staple for the treatment of relapsed/refractory multiple myeloma. Unfortunately, further success of bortezomib has been hampered by tumor resistance (both intrinsic and acquired), severe toxicities, and low efficacy in solid tumors. To overcome these limitations, especially resistance, scientists have investigated the molecular mechanisms involved and developed novel strategies to improve proteasome inhibitor-based therapies and patient care. By improving chemical and biochemical properties, binding affinity and reversibility, potency and selectivity, several second-generation proteasome inhibitors have been developed, among them carfilzomib, which is more specific and less toxic than bortezomib, and has become the second FDA-approved proteasome inhibitor for multiple myeloma treatment. Other cutting-edge strategies to overcome bortezomib resistance include selectively targeting immunoproteasomes or sites outside the catalytic core (such as 19S deubiquitinases or ubiquitin E3 ligases) and developing novel combinational therapies. Definitively elucidating the mechanisms responsible for proteasome inhibitor resistance is key in designing new compounds to fully overcome this resistance.

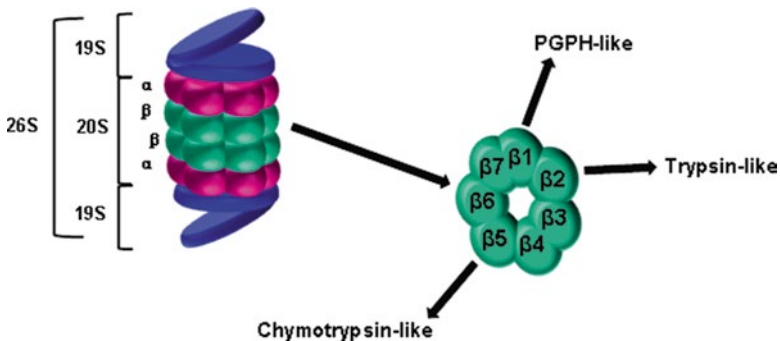
## 1.2 Ubiquitin–Proteasome Pathway

The UPP is the major pathway responsible for regulating protein turnover in cells. The UPP is so critical to normal cellular function that its discoverers, Aaron Ciechanover and Avram Hershko, were awarded the 2004 Nobel Prize in Chemistry [1, 2]. Proteins degraded by the UPP are involved in many biological processes, including development, differentiation, proliferation, signal transduction, and apoptosis [3]. In addition to its critical role in protein homeostasis, the proteasome also functions in several non-proteolytic processes, such as transcription initiation and elongation [4], regulation of gene expression [5], and transcription-coupled nucleotide excision repair [6].

Protein degradation is carried out via two distinct steps: (1) conjugation of multiple ubiquitin molecules to the protein substrate and (2) degradation of the ubiquitin-tagged substrate by the 26S proteasome (Fig. 1.1) [7]. The 26S proteasome is a large (2.5 MDa), multi-subunit complex that is localized both in the cytosol and nucleus of cells [8–10]. The 26S proteasome is made up of the catalytic 20S core and one or two 19S regulatory caps (Fig. 1.2) [11, 12]. The 20S core is comprised of 28 subunits that form a barrel-like structure of four alternately stacked rings: two  $\alpha$ -rings surrounding two  $\beta$ -rings, each with seven subunits [13–15]. The role of the  $\alpha$ -subunits is to allow only unfolded proteins to enter the 20S core, while the  $\beta$ -subunits are responsible for the proteolytic activities of the proteasome, which are dependent on an amino-terminal nucleophilic Thr1 residue [15]: caspase or peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like activity, carried out by  $\beta$ 1, trypsin-like by  $\beta$ 2 and chymotrypsin (CT)-like by  $\beta$ 5 (Fig. 1.2) [15–17]. The 19S regulatory caps (700 kDa) can be divided into a base and a lid (Fig. 1.2); the base is responsible for the recognition and unfolding of ubiquitinated protein substrates, as well as



**Fig. 1.1** The ubiquitin–proteasome pathway. There are two steps in the UPP: ubiquitination and target degradation. The ubiquitination step is carried out by three distinct types of enzymes, E1 (ubiquitin activating), E2s (ubiquitin conjugating), and E3s (ubiquitin ligating). First, ubiquitin is activated by E1, the activated ubiquitin is transferred to an E2 for conjugation, and finally, an E3 ubiquitin-ligating enzyme aids in the transfer of active ubiquitin to lysine residues within the target protein. The target protein is then recognized, deubiquitinated, and translocated to the 26S proteasome by components of the 19S regulatory cap, followed by degradation into small peptide fragments, and the ubiquitin molecules are recycled



**Fig. 1.2** Proteasome structure. The 26S constitutive proteasome is comprised of a 20S catalytic core and one or two 19S regulatory caps. The 20S core contains four stacked rings—two α-rings surrounding two β-rings, each consisting of seven subunits. The catalytic activity is carried out by three β-subunits: β1, β2, and β5 responsible for caspase or peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like, trypsin-like, and chymotrypsin (CT)-like, respectively

opening the 20S pore and transport of protein substrates into the core, while the major responsibility of the lid component is deubiquitination of substrates before degradation. The base contains six ATPase subunits, Rpt1–6, which form a hexameric ring [18–20], as well as two non-ATPase subunits Rpn-1 and Rpn-2 [21, 22], and the lid consists of at least six non-ATPases, including Rpn-10/S5a and Rpn-13/Adrm1, which contain ubiquitin-interacting motifs (UIMs) [23]. Rpn-10/S5a has two UIMs that preferentially binds poly-ubiquitinated substrates [24], and Rpn-13/Adrm1 binds to the non-ATPase Rpn-2 to recruit deubiquitinating enzymes (DUBs) to the proteasome [25–27]. Deubiquitination is very highly regulated and is important for recycling ubiquitin molecules and controlling the rate of ubiquitin-dependent proteasomal degradation [27].

The ubiquitination step of the UPP is carried out by three distinct types of enzymes, E1 (ubiquitin activating), E2s (ubiquitin conjugating), and E3s (ubiquitin ligating). The first step in the pathway is ATP-dependent E1-mediated activation of ubiquitin, a small 76-amino-acid protein that is expressed ubiquitously and serves as a tag for protein substrates destined for various fates, including membrane trafficking, protein kinase activation, DNA repair and chromatin remodeling, as well as degradation by the UPP (Fig. 1.1) [28]. Activated ubiquitin is then transferred from E1 to an E2 enzyme, a group of enzymes responsible for ubiquitin conjugation, and then to an E3 ubiquitin-ligating enzyme, which aids in the transfer of active ubiquitin to lysine residues within the target protein (Fig. 1.1) [29, 30]. Following the conjugation of a sufficiently sized ubiquitin chain, which is four in most cases, except in rare cases such as mODC and HIF-1 $\alpha$ , which require no ubiquitination for proteasomal degradation [31–33], the protein substrate is recognized, deubiquitinated, and translocated to the 26S proteasome by components of the 19S regulatory cap [34, 35]. Finally, the substrate is degraded into small peptide fragments and the ubiquitin molecules are recycled (Fig. 1.1) [36]. This process is tightly controlled and extremely crucial in the regulation of many cellular processes, including those involved in tumorigenesis [37], which makes it a promising target for anticancer therapeutic agents.

Because the UPP plays such a crucial role in normal cellular function, it is no surprise that it has also been implicated in the development, growth, and survival of various malignancies [38]. Thus, targeting factors involved in the synthesis and degradation of proteins, including the UPP, has been explored as a potential anticancer strategy [39]. Several studies have reported increased proteasome activity in various cancers, including colon, prostate, and leukemia [40–42], indicating that cancer cells may be more dependent on the UPP than normal cells and that targeting this pathway in the treatment of human cancer is a promising strategy. Specifically, inhibition of chymotrypsin (CT)-like activity has been associated with cell cycle arrest and apoptosis [43, 44], indicating that proteasome inhibition may effectively cause selective cell death in cancer cells, as well as sensitizing them to chemotherapeutics [45], with little toxicity in normal cells. Importantly, the use of proteasome inhibitors was validated by the US Food and Drug Administration (FDA) approval of bortezomib for the treatment of relapsed and refractory multiple myeloma and mantle cell lymphoma.

## 1.3 Proteasome Inhibitors

### 1.3.1 Early Inhibitors

Prior to the development and approval of bortezomib, numerous preclinical studies were carried out to validate the UPP as a valid druggable target. The most widely investigated early inhibitors include the peptide aldehydes, which are analogs of proteasome substrates that inhibit the CT-like activity of the proteasome and include MG-132 (Cbz-leu-leu-leucinal), MG-115 (Cbz-leu-leu-norvalinal), and ALLN (acetyl-leu-leu-norleucinal) [46, 47]. Importantly, in-depth studies using these complexes aided in elucidating the active site for compounds in this class, X-ray diffraction revealed that ALLN forms a hemiacetal complex with the N-terminal threonine hydroxyl groups of the catalytic  $\beta$ -subunits [14, 48]. Another peptide aldehyde inhibitor, PSI (Cbz-ile-glu(O-t-Bu)-alaleucinal), has been shown to suppress 26S proteasome-mediated proteolysis without affecting isopeptidase or ATPase activities [49]. These inhibitors are extremely potent (MG-132  $K_i$  = low nanomolar in purified proteasome;  $IC_{50}$  = low micromolar in cultured cells) and their inhibitory activities are reversible by their removal from the system [46, 47]. Interestingly, because peptide aldehydes are also able to inhibit calpains and some lysosomal cysteine proteases, certain degradative processes that were originally believed to be carried out by calpains were shown to actually be proteasomal processes.

Vinyl sulfone peptides have also been reported to be potent inhibitors of the proteasome in cell models [50]. These peptides exert their proteasome-inhibitory activity through covalent binding to the hydroxyl groups of the active site threonine within the  $\beta$ -subunits, and their use in human lymphoma cells resulted in proteasome inhibition followed by the appearance of distinct cell variants expressing a compensatory proteolytic system, which has not been clearly identified [51].

Other early inhibitors of the proteasome include lactacystin and its derivative *clasto*-lactacystin  $\beta$ -lactone, the active form to which it is converted in aqueous solution [52]. These are naturally occurring products that differ structurally from the peptide aldehydes and are much more specific. Lactacystin was first isolated from actinomycetes because of its ability to promote neurite outgrowth and block cell division in cultured neurons [53]. These compounds have a mode of action similar to that of the vinyl sulfones [54, 55].

Other naturally occurring metabolites that have been used in the preclinical setting as inhibitors of the proteasome include TMC-95A and argyrin A. TMC-95A is a cyclic tripeptide that was isolated from *Apiospora montagnei*. TMC-95A specifically binds via hydrogen bonds to all three catalytic  $\beta$ -subunits and causes inhibition in the low nanomolar range [56, 57]. The tumor growth suppression caused by argyrin A, a cyclic octapeptide derived from *Archangium gephyra*, has been attributed to the inhibition of proteasomal degradation of p27<sup>kip1</sup> CDK inhibitor [58, 59]. Following the identification of these inhibitors, many other compounds were identified and designed to specifically target the tumor proteasome, ultimately resulting in the USFDA approval of bortezomib in 2003.

### ***1.3.2 Bortezomib, the First Clinically Approved Proteasome Inhibitor in Preclinical Studies***

Bortezomib (Velcade®) is a dipeptide boronic acid derivative that was first synthesized in 1995 by Myogenics Company and contains pyrazinoic acid, phenylalanine, and leucine in its structure. Bortezomib showed considerable apoptosis-inducing activity in a variety of tumor cell lines and animal models [60–62], and in 2003, seven years after its initial synthesis, bortezomib was approved by the USFDA for the treatment of relapsed multiple myeloma, and in 2006, it was approved for the treatment of mantle cell lymphoma. Bortezomib is able to enter nearly all tissues except brain and adipose, and is able to distribute to the plasma within 10 min of IV injection [63–66]. Furthermore, bortezomib is metabolized through intracellular cytochrome p450-mediated oxidative deboronation [67] and its half-life is more than 40 h [65].

Bortezomib is a reversible inhibitor of the 26S proteasome, with proteasome activity generally recovering within 72 h of administration [68]. Binding of the boronic acid group in bortezomib to the threonine hydroxyl group in the active site of the  $\beta 5$  subunit results in proteasome inhibition and, ultimately, cell death [69]. Bortezomib has been successful in hematological malignancies, but less than encouraging results have been observed in solid tumors [70, 71], limiting its use in the clinic.

Several preclinical studies demonstrated the potency of bortezomib against human tumor cells in vitro and in in vivo xenograft animal models. A standard NCI-60 screen revealed that bortezomib could potently inhibit cell proliferation [60] and induce apoptosis in many malignant cell lines, including multiple myeloma, prostate, pancreatic, renal and squamous cell carcinomas [72–77]. Importantly, the anti-tumor activity of bortezomib was observed in both chemoresistant and chemosensitive myeloma cells, and the sensitivity of resistant cells to chemotherapy was increased significantly when combined with a sublethal dose of bortezomib with no effect on normal hematopoietic cells [78, 79]. Additionally, in an in vitro study of four ovarian and three prostate cancer cell lines, bortezomib had comparable effects on cells derived from solid tumors and hematological malignancies [61]. Bortezomib was also able to potently inhibit the growth of multiple myeloma xenografts in mice [80].

Multiple targets of bortezomib have been identified in malignant cells, including the NF- $\kappa$ B signaling pathway. NF- $\kappa$ B is a p50/p65 heterodimer that usually exists in an inactive form in the cytoplasm bound to its inhibitory protein, I $\kappa$ B, and upon degradation of I $\kappa$ B, the NF- $\kappa$ B complex is activated and can translocate into the nucleus where it stimulates transcription of various genes including cytokines (IL-6, TNF- $\alpha$ ), survival factors (IAPs, Bcl-X<sub>L</sub>), and insulin-like growth factor 1 (IGF-1), ultimately resulting in proliferation, resistance to apoptosis, and drug resistance in cancer cells [81]. Bortezomib is able to prevent degradation of I $\kappa$ B, blocking activation of NF- $\kappa$ B and suppressing expression of related cytokines and survival factors in drug-resistant multiple myeloma cells expressing increased NF- $\kappa$ B activity [78, 82]. In contrast, other studies have shown that the NF- $\kappa$ B pathway may not be important in bortezomib-mediated tumor cell death. Specifically, in a study of mice bearing human multiple myeloma cells, treatment with bortezomib was associated with NF- $\kappa$ B activation, rather than inhibition [83].

Another possible target of bortezomib is NOXA (Latin for damage) [84], a pro-apoptotic member of the Bcl-2 family [85] that is involved in p53-mediated apoptosis, gene expression of which is associated with direct activation of its promoter by p53 [85]. Thus, upregulation of p53 and subsequent *Noxa* gene expression may be one mechanism of chemo- or radiotherapy-induced apoptosis. Studies have shown that NOXA upregulation induces apoptosis through interaction with, and inhibition of anti-apoptotic Bcl-xL and Bcl-2 proteins, or through stimulation of other pro-apoptotic factors [86, 87]. Importantly, bortezomib treatment in myeloma and melanoma cell lines resulted in p53-independent induction of NOXA and blockade of NOXA with an antisense oligonucleotide caused only 30 % to 50 % reduction in bortezomib-induced apoptosis [84]. Bortezomib induces NOXA in various p53-defective tumor cell lines [88], and clinical studies indicate that bortezomib suppresses tumor growth in a p53-independent manner [11, 89]. Importantly, NOXA induction by bortezomib is selective to cancer cells over normal cells, with levels unaffected in normal melanocytes [84, 90, 91].

Still other mechanisms of bortezomib-mediated apoptosis include inhibition of angiogenesis in human myeloma, pancreatic and squamous cell cancer xenografts [77, 92]; induction of endoplasmic reticulum (ER) stress and generation of reactive oxygen species (ROS) [93, 94]; induction of extrinsic and intrinsic apoptotic pathways via activation of caspase-8 and caspase-9 [95, 96]; activation of the p38 mitogen-activated protein kinase (MAPK) pathway [97]; and disruption of the interaction between tumor cells and dendritic cells [98]. Multiple targets generally play important roles in bortezomib-mediated apoptosis in some cancer cells, while different targets may be critical in other cells.

### ***1.3.3 Bortezomib in Clinical Trials***

#### **1.3.3.1 Phase I/II Trials**

The promising preclinical data involving bortezomib resulted in a series of clinical trials that ultimately led to the USFDA approval of bortezomib as a treatment for multiple myeloma. One phase I trial of 27 patients with relapsed multiple myeloma investigated bortezomib as a single agent, and found that bortezomib induced a dose-dependent inhibition of 20S proteasome activity [99], confirming preclinical findings that bortezomib could inhibit proteasome activity in a dose- and time-dependent manner. Two other phase I studies evaluated bortezomib in combination with doxorubicin. In the first, 42 patients with advanced hematologic malignancies were enrolled to obtain preliminary response data and to determine the maximum tolerated dose (1.30 mg/m<sup>2</sup>) and dose-limiting toxicities (fatigue, thrombocytopenia, lymphopenia, nausea, constipation, peripheral neuropathy, and anemia) [100]. The other enrolled 22 patients with multiple myeloma, with eight patients achieving complete response (36 %) or near-complete response, and another eight partial responses (36 %) [100].



Additional phase I trials have investigated the effects of bortezomib either alone or in combination in solid tumors. Single-agent bortezomib showed antitumor activity in patients with advanced androgen-independent prostate cancer [101], but no significant responses were observed in patients with advanced metastatic breast cancer or neuroendocrine tumors [102, 103]. In combination with carboplatin, an overall response rate of 47 % was observed in recurrent ovarian or primary peritoneal cancer patients, but in combination with either docetaxel [104] or prednisone [105], hormone refractory and castrate-resistant metastatic prostate cancer patients achieved no significant responses. Therefore, while bortezomib has shown promise in hematological malignancies, it has proven quite ineffective against solid tumors.

The general success of phase I trials led to several phase II trials. In the SUMMIT (Study of Uncontrolled Multiple Myeloma Managed with Proteasome Inhibition Therapy) trial, 202 patients with relapsed or refractory myeloma with prior treatment were treated with 1.3 mg/m<sup>2</sup> bortezomib on days 1, 4, 8, and 11 of a 3-week cycle for as many as eight cycles, and an overall response rate of 35 % was observed [106]. In the CREST (Clinical Response and Efficacy Study of Bortezomib in the Treatment of Relapsing Multiple Myeloma) trial, 67 patients with relapsed/refractory multiple myeloma were randomly divided to receive either 1.0 or 1.3 mg/m<sup>2</sup> of bortezomib. The study ultimately showed that bortezomib was effective in relapsed multiple myeloma patients at a lower dose of 1.0 mg/m<sup>2</sup> [107].

Two other phase II trials examined bortezomib in combination with other agents. One study reported a 95 % response rate in relapsed multiple myeloma patients treated with a combination of bortezomib, dexamethasone, and doxorubicin [108]. Another study in patients with symptomatic multiple myeloma with no prior treatment compared single-agent bortezomib to bortezomib in combination with dexamethasone. Of the 32 patients, 22 were treated with the combination, and an increased response was seen in 15 of 22 patients (68 %) [45].

Additionally, the effects of bortezomib against mantle cell lymphoma and non-Hodgkin's lymphoma have also been investigated in clinical trials. A trial of patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma showed a 58 % overall response rate as a result of bortezomib treatment [109]. Another study conducted in patients with pretreated and untreated mantle cell lymphoma revealed response rates of 46.2 % and 46.7 %, respectively, following treatment with 1.3 mg/m<sup>2</sup> bortezomib, suggesting that bortezomib is an effective treatment for mantle cell lymphoma [110]. Finally, no significant response or survival advantage was observed in another phase II study evaluating the use of bortezomib and pemetrexed alone or in combination in advanced NSCLC with prior treatment, but bortezomib was better tolerated when given in combination with pemetrexed [111]. More clinical trials are being conducted to further explore the use of bortezomib in NSCLC. Unfortunately, phase II trials investigating the efficacy of bortezomib in solid tumors have yielded disappointing results.

### 1.3.3.2 Phase III Clinical Trials

Based on phase II trial results, a large international phase III trial in relapsed multiple myeloma patients with 1–3 prior therapies compared the effects of bortezomib to high-dose dexamethasone [75]. Patients ( $n=669$ ) received either 1.3 mg/m<sup>2</sup> bortezomib (twice weekly for 2 weeks followed by a 1-week rest, intravenously) or high-dose dexamethasone (40 mg orally). Patients receiving bortezomib had a combined complete and partial response rate of 38 % compared to 18 % for the dexamethasone-treated patients, with median times to progression of 6.22 months in the bortezomib group versus 3.29 months in the dexamethasone group. Among patients taking bortezomib, the median time to progression was 6.22 months and 1-year survival rate was 80 %, while that for patients taking dexamethasone was 3.29 months and 66 % [75], demonstrating the advantage of bortezomib over dexamethasone in terms of response rate, time to progression, and survival.

Another study included 638 relapsed/refractory multiple myeloma patients who received 1.3 mg/m<sup>2</sup> bortezomib and achieved an overall response rate of 67 % [112]. After completion of at least two cycles for progressive and four cycles for stable disease, 20 mg/day dexamethasone was added on the day of and after each bortezomib dose. Of the patients receiving dexamethasone, enhanced response was observed in 34 %, suggesting that bortezomib, alone or in combination with dexamethasone, is both safe and effective for the treatment of relapsed/refractory multiple myeloma in patients with prior treatment [112]. The APEX (Assessment of Proteasome Inhibition for Extending Remissions) trial assessed the impact of dose modification on the severity and reversibility of peripheral neuropathy associated with bortezomib treatment in patients with relapsed multiple myeloma [113]. Peripheral neuropathy could be improved by dose modification without adverse effects on the outcome in 37 % of patients (124/331) following several cycles of bortezomib treatment [113], indicating that bortezomib-induced peripheral neuropathy is not only manageable, but also reversible in most relapsed myeloma patients.

The efficacy of bortezomib in combination with conventional chemotherapeutics was conducted at 151 centers in 22 countries. Patients with untreated multiple myeloma ( $n=682$ ) were randomized to receive either a combination of bortezomib plus melphalan–prednisone or melphalan–prednisone alone [114]. Results revealed that bortezomib plus melphalan–prednisone may be a valuable frontline treatment option for myeloma patients [114]. Most recently, in the VISTA trial, bortezomib plus melphalan and prednisone was compared to melphalan and prednisone alone in multiple myeloma patients with no previous treatment. A prolonged follow-up (median=36.7 months) indicated that bortezomib-based drugs as first-line treatments afford greater survival advantage than treatment with conventional drugs followed by salvage with bortezomib-based treatments [115]. Additionally, initial treatment with bortezomib, compared to initial treatment with traditional chemotherapeutics, resulted in less resistance to later therapies [115]. Overall, preclinical and clinical data evaluating the efficacy and safety of bortezomib have shown that the use of proteasome inhibitors as anticancer agents is a promising strategy that should be further investigated.

However, while bortezomib is successful in the clinic, toxicities and resistance have been reported, suggesting that further development of drugs like bortezomib is necessary. In fact, some second-generation proteasome inhibitors [116, 117] with different properties have been developed, with one, carfilzomib, being FDA approved. Additionally, inhibitors that specifically target the immunoproteasome (immunoproteasome-specific inhibitors, IPSIs) [117], as well as natural compounds that are able to inhibit the proteasome, may be sufficiently potent with significantly less adverse effects than currently approved drugs [118]. The use of these novel inhibitors may aid in overcoming bortezomib resistance or sensitizing resistant cells to bortezomib treatment, which could potentially result in increased clinical success.

### ***1.3.4 The Second Clinically Approved Proteasome Inhibitor, Carfilzomib***

Following the clinical success of bortezomib, the second-in-class proteasome inhibitor carfilzomib (Kyprolis<sup>®</sup>) was granted accelerated approval by the USFDA in July 2012 for the treatment of patients with MM progressing on or after treatment with bortezomib and an immunomodulatory agent. Carfilzomib is a peptide epoxyketone related to epoxomicin [119] that irreversibly inhibits the CT-like activity of the proteasome with high selectivity [120]. Preclinical studies revealed that carfilzomib inhibits CT-like activity in both the constitutive proteasome and the inducible immunoproteasome with  $IC_{50}$  values of 6 and 33 nM, respectively [121]. Carfilzomib was also extremely effective at suppressing tumor growth in cultured cell and tumor xenograft models, with prolonged proteasome inhibition for longer than one week in mice [121]. Importantly, carfilzomib was active against bortezomib-resistant cultured myeloma and patient plasma cells [119].

#### **1.3.4.1 Phase I/II Trials**

The data observed in cultured cell and xenograft models led to a series of clinical trials investigating the properties and efficacy of carfilzomib. In one phase I study, carfilzomib was administered on consecutive days twice weekly in patients with relapsed and refractory multiple myeloma or lymphoma. One hour following IV administration of 27 mg/m<sup>2</sup> carfilzomib, CT-like activity in whole blood and peripheral blood mononuclear cells (PBMCs) was inhibited by approximately 85 % and 90 % on average, respectively, and this inhibition was sustained throughout the trial [122]. Another small phase I dose-escalation study evaluated the safety and efficacy of carfilzomib in relapsed or refractory myeloma and lymphoma, with patients ( $n=29$ ) receiving carfilzomib for five consecutive days within 14-day cycles [123]. One unconfirmed complete response, one partial response, and two minimal responses were observed with observable antitumor activity at or above 11 mg/m<sup>2</sup> and a maximum tolerated dose of 15 mg/m<sup>2</sup>. Grade 1–2 toxicities included nausea,

diarrhea, and fatigue in more than one-third of patients. At the highest dose administered ( $20 \text{ mg/m}^2$ ), grade 3 febrile neutropenia and grade 4 thrombocytopenia were reported, and no grade 3 or 4 peripheral neuropathies were reported [123]. An additional phase I/II study investigated the tolerability, efficacy, and pharmacokinetic and pharmacodynamic profiles of carfilzomib in advanced solid tumors [124]. Carfilzomib was administered IV twice weekly on consecutive days within 28-day cycles. A small group of patients ( $n=14$ ) received carfilzomib during the phase I dose escalation, and the single dose-limiting toxicity was determined to be grade 3 fatigue at the highest administered dose ( $36 \text{ mg/m}^2$ ). The maximum planned dose was determined based on the phase I cohort, and 65 additional patients then received carfilzomib at the maximum planned dose in a phase II study. The most common side effects were fatigue, nausea, anorexia, and dyspnea. No hepatotoxicity or  $\geq$ grade 2 peripheral neuropathy was reported. The half-life was determined to be  $<1$  h, and one hour post treatment on day one of cycle two, proteasome CT-like activity in whole blood and PBMCs was inhibited by  $\geq 80\%$ . Importantly, partial responses were reported in two patients (14%) in the phase I study, with 21.5% stable disease after four cycles in evaluable patients ( $n=51$ ) in the phase II cohort [124].

Another phase I/II trial included patients with newly diagnosed multiple myeloma ( $n=53$ ) and evaluated the efficacy and tolerability of the CRd combination treatment (carfilzomib + lenalidomide + dexamethasone) [125]. Carfilzomib was administered at 20, 27, or  $36 \text{ mg/m}^2$  twice weekly on consecutive days, lenalidomide was given at a dose of 25 mg/day daily for the first 21 days, and weekly dexamethasone was given at 40 mg during cycles 1–4 and 20 mg during any additional cycles. The maximum planned dose (carfilzomib  $36 \text{ mg/m}^2$ ) was expanded in the phase II study. Toxicities (grade 3–4) included anemia, thrombocytopenia, and neutropenia, hypophosphatemia, and hyperglycemia; no grade 3–4 peripheral neuropathy was observed and dose modification was not required in a majority of patients. A near-complete response was reported in 62%, and complete response occurred in 42% of patients ( $n=53$ ) after an average of 12 cycles. After a median follow-up of 13 months, the 24-month progression-free survival estimate was 92% [125]. Thus, the combination of carfilzomib, lenalidomide, and dexamethasone is highly effective and well tolerated in treatment-naïve multiple myeloma patients.

Finally, a single-arm multicenter phase II was completed in relapsed/refractory multiple myeloma patients ( $n=46$ ) with at least two prior therapies [126]. Patients were given  $20 \text{ mg/m}^2$  carfilzomib IV on consecutive days twice weekly every 28 days for up to 12 cycles. The overall response and clinical benefit response rates were 16.7% and 23.8%, respectively, in the 42 evaluable patients, with seven partial responses. Median durations of response were 7.2 months and 13.8 months, respectively. Anemia, fatigue, and thrombocytopenia were the most common treatment-related adverse effects, and events of neuropathy were rare [126]. The promising results of this pilot study resulted in an amendment to test a higher dose in additional patients (PX-171-003-A1). During the PX-171-003-A1 study [127], patients ( $n=266$ ) received single-agent carfilzomib  $20 \text{ mg/m}^2$  IV twice weekly for 3

of 4 weeks in cycle one, followed by 27 mg/m<sup>2</sup> for the remaining cycles (maximum = 12). The overall response rate (at least partial response) was 23.7 % with median duration of response of 7.8 months and median overall survival of 15.6 months. Manageable toxicities included anemia, nausea, fatigue, and thrombocytopenia, with grade 1–2 peripheral neuropathy in 12.4 % of evaluable patients [127]. Patients in this study had an average of five prior treatments, but the responses observed in this trial were quite durable and indicated that carfilzomib may be clinically beneficial in patients who fail on other chemotherapeutics, and based on this study, carfilzomib was approved by the FDA in July 2012 for relapsed and refractory multiple myeloma and mantle cell lymphoma.

### 1.3.4.2 Phase III Trials

Based on the promising phase I/II results, a randomized phase III study, FOCUS (Carfilzomib for Advanced Refractory Multiple Myeloma European Study), is being conducted to compare overall survival following single-agent carfilzomib treatment with best supportive care treatments in relapsed and refractory multiple myeloma patients who have received at least three previous treatments [128]. Enrolled patients ( $n \approx 300$ ) have responded to at least one prior therapy and are refractory to their most recent therapy. Patients were randomized to receive either IV carfilzomib, 20 mg/m<sup>2</sup> on days 1–2 of cycle one, escalating to 27 mg/m<sup>2</sup> on days 8, 9, 15, and 16 for the remaining cycles (up to 16), or an active BSC regimen consisting of corticosteroid treatment of prednisolone 30 mg, dexamethasone 6 mg, or equivalent every other day with optional oral cyclophosphamide 50 mg once daily. Treatment will continue until disease progression or unacceptable adverse events occur. The primary endpoint will be overall survival with secondary endpoints of progression-free survival, overall response rate, and safety [128]. Enrollment has been completed and the study has begun with anticipated completion in 2015 [NCT01302392; Onyx Therapeutics, Inc.].

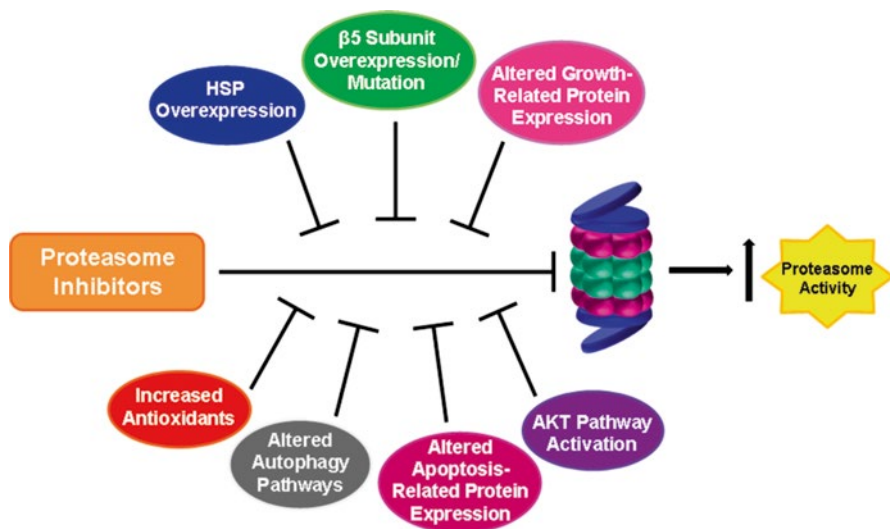
Another randomized phase III, open-label, multicenter study is comparing CRd (carfilzomib + lenalidomide + dexamethasone) and Rd (lenalidomide + dexamethasone) regimens in patients with relapsed multiple myeloma [NCT01080391; Onyx Therapeutics, Inc.]. Approximately 750 patients have been randomized to receive either Rd (40 mg oral dexamethasone on days 1, 8, 15, and 22 + 25 mg oral lenalidomide on days 1–21 in 28-day cycles) or CRd (20 mg/m<sup>2</sup> or 27 mg/m<sup>2</sup> IV carfilzomib + 40 mg oral dexamethasone on days 1, 8, 15, and 22 + 25 mg oral lenalidomide on days 1–21 in 28-day cycles; carfilzomib will be discontinued after completion of 18 cycles). Progression-free survival is the primary endpoint of this study, and overall survival, overall response rate, response duration, disease control, safety, time to progression, and quality of life are secondary endpoints. Severity and incidence of adverse effects will also be compared between the two treatment regimens. This study is expected to be completed in early 2014 [NCT01080391; Onyx Therapeutics, Inc.].

## 1.4 Resistance to Proteasome Inhibitors

Unfortunately, although clinical success has been achieved with proteasome inhibitors, resistance has emerged as a limiting factor in their continued clinical use. Resistance to proteasome inhibitors, as well as other drugs, can be either inherent or acquired. Inherent resistance is resistance which exists within cells without any exposure to a drug. This type of resistance is fairly uncommon in cancer, but has been reported in regard to antibiotics. Acquired resistance occurs following exposure to a drug, generally by genetic mutations and overexpression of target proteins. The exact mechanisms by which cells become resistant to proteasome inhibitors have yet to be fully elucidated, but several studies have explored potential mechanisms (Fig. 1.3).

### 1.4.1 Inherent Resistance

In a study of relapsed/refractory acute leukemia patients who had progressed on prior treatments, bortezomib treatment resulted in minimal responses [129], suggesting the potential for inherent resistance to proteasome inhibitors due to prior treatments. The ECOG E2A02 trial conducted with newly diagnosed high-risk



**Fig. 1.3** Potential mechanisms of proteasome inhibitor resistance. Several factors have been implicated in resistance to proteasome inhibitors. These include overexpression or mutation of  $\beta 5$  subunits, overexpression of heat shock proteins (HSPs), AKT pathway activation, altered expression of apoptosis- and growth-related proteins, altered autophagy pathways, and increased antioxidants

multiple myeloma patients showed no clinical response following single-agent bortezomib treatment [130]. Bortezomib also failed to show clinical benefit in several other hematological and solid tumors [129]. The lack of benefit from bortezomib as an initial treatment suggests that some tumors may simply be inherently resistant to treatment with proteasome inhibitors.

## 1.4.2 Acquired Resistance

While little is known about inherent resistance to proteasome inhibitors like bortezomib, a number of cell-based studies have elucidated putative mechanisms of acquired resistance either at the proteasome level or its downstream effectors. These include overexpression or mutation of the proteasomal  $\beta 5$  subunit, upregulation of heat shock proteins (HSPs), altered expression of apoptosis-related proteins, AKT pathway activation, overexpression of other growth-related proteins, altered autophagy pathways, and increased antioxidant levels (Fig. 1.3).

### 1.4.2.1 $\beta 5$ Subunit Overexpression/Mutation

When human monocytic/macrophage THP1 cells were treated with increasing concentrations of bortezomib, up to 60-fold overexpression of proteasomal  $\beta 5$  subunit (PSMB5) protein was observed. Additionally, the overexpressed  $\beta 5$  contained an alanine–threonine mutation at position 49 in the highly conserved bortezomib-binding pocket [131]. Together the overexpression and mutation resulted in resistance to bortezomib as well as cross-resistance to  $\beta 5$ -targeted cytotoxic peptides 4A6, MG132, MG262, and ALLN [131]. Interestingly, there were no marked changes in the baseline CT-like activity, and when the *PSMB5* gene was silenced by siRNA, its sensitivity to bortezomib was restored [131]. A different set of mutations in the *PSMB5* protein at positions 49 and 50, including Ala49Val, Ala49Thr, and Ala50Val, as well as the Ala49Thr mutation, were reported in T lymphoblastic lymphoma/leukemia cells developed from the Jurkat cell line when treated with increasing concentrations of bortezomib [132]. Similarly, human leukemia K562 cells have been shown to be more resistant to bortezomib compared to other leukemia and myeloma cell lines due to inherent overexpression of proteasomal  $\beta 5$ . However, there is no direct evidence that these phenomena, i.e., mutations or  $\beta 5$  overexpression, are responsible for bortezomib resistance in vivo [133]. In fact, a multiple myeloma patient who rapidly developed resistance to bortezomib (evident by sudden and accelerated disease progression and death) had no mutations in the *PSMB5* coding region, indicating that there may not be a correlation between bortezomib resistance and  $\beta 5$  mutation [134]. Further in-depth, large-scale studies are warranted to determine the role of  $\beta 5$  mutations and overexpression in in vivo resistance to proteasome inhibitors.

### 1.4.2.2 Upregulation of HSPs

The heat shock proteins are important in mediating resistance to apoptosis [135, 136], and many HSPs, especially HSP-72, are upregulated following proteasome inhibition. Several studies have reported dramatic HSP-72 upregulation resulting from treatment with a variety of proteasome inhibitors, including tripeptidyl aldehyde proteasome inhibitors, lactacystin and MG-132 [137–139]. Some studies demonstrated that proteasome inhibitor-mediated upregulation of HSP-72 was pro-apoptotic [138, 139], while others showed that MG-132 treatment caused an increase in HSP-72 expression and suppressed JNK activation, preventing JNK-mediated apoptosis by subsequent heat stress [139]. HSP-72 upregulation as a mechanism of proteasome inhibitor resistance was validated by a report showing that blocking HSP-72 by the introduction of an antisense oligonucleotide potentiated the apoptosis-inducing ability of MG-132 [140]. More recently, HSP-72 knockdown via siRNA was also shown to potentiate MG-132-induced cell death in prostate cancer cells [141].

Other HSP family members may also be involved in acquired resistance to proteasome inhibitors. Gene profiling of myeloma cells following bortezomib treatment revealed that several other HSPs are also induced by proteasome inhibition, including HSP-27, HSP-70, and HSP-90 [95, 142, 143]. One group demonstrated that bortezomib promotes increased phosphorylation of HSP-27 through activation of p38 and used p38 inhibitors and antisense-mediated downregulation of HSP-27 to reverse proteasome inhibitor resistance [142, 144]. HSP-70 has also been implicated in bortezomib resistance [143], and the flavonoid quercetin has been shown to inhibit HSP-70 mRNA and protein expression [145], suggesting that knockdown or inhibition of HSP-70 may also reverse acquired bortezomib resistance. Importantly, HSP-70 expression is high in pancreatic cancer cells, and inhibition of HSP-70 via quercetin treatment and siRNA knockdown both induced apoptosis *in vitro* [146].

Finally, HSP-90, which mediates the correct folding of various signal transduction intermediates, has also been implicated in proteasome inhibitor resistance [95]. In fact, synergistic cell death was observed in breast cancer cells treated with the combination of bortezomib and an HSP-90 inhibitor [147]. Additionally, in several preclinical multiple myeloma cell models, the combination resulted in increased apoptosis [147–149], but in pancreatic cancer the cell death resulting from combination treatment appears to be necrotic rather than apoptotic [150], indicating that the combination of HSP-90 antagonists with proteasome inhibitors must be further evaluated to more clearly understand their interactions with one another.

### 1.4.2.3 Altered Expression of Apoptosis-Related Proteins

As discussed previously, the Bcl-2 family members Bim [151] and Noxa [88] have been implicated in proteasome inhibitor-induced cell death in some cell types. While mutations causing inactivation of these proteins are rare in tumors [152, 153], cells



may acquire resistance to proteasome inhibitors via epigenetic mechanisms. In fact, miR-17-92 and NFB2/p52 have recently been reported to repress Bim expression [154, 155], and Bmi-1-dependent methylation has been linked to Noxa expression [156]. The effects of Noxa and Bim could also be abrogated by overexpression of anti-apoptotic Bcl-2 proteins [157]. Small molecule inhibitors targeting Bcl-2, Bcl-xL (ABT-737), and MCL-1 (obatoclax) have been shown to significantly enhance bortezomib-induced cell death in various human cancer cell lines [158–160].

Other proteins that contribute to cell death, like p27, have also been reported to increase following proteasome inhibition [59, 161], and although inactivation of p27 through mutations is uncommon, its expression is often decreased due to increased Skp2 activity and proteasome-mediated degradation [162]. Methylation of p27 gene promoter occurs in almost 10 % of cancers [163], and proteasome inhibitor-resistant tumors may display increased methylation patterns that could contribute to the resistant phenotype. Additionally, p27 can be phosphorylated by AKT [164], which causes changes in its subcellular localization [164, 165], also potentially contributing to acquired proteasome inhibitor resistance.

#### 1.4.2.4 Akt Pathway Activation

The pro-survival PI3K/Akt pathway is constitutively active in many cancers, and several pathways have been implicated in Akt activation, including amplification of PI3K [166] or Akt [167], growth factor receptor signaling [168], PTEN deletion [169], or mutation of Ras family members [170]. Akt activation, both constitutive and induced, can impair the activity of bortezomib [95, 171, 172]. Bortezomib can also directly activate Akt in some cell lines [173], and Akt inhibitors (both direct and indirect), including the PKC antagonist enzastaurin [174], PI3K inhibitors like perifosine, and the Raf inhibitor sorafenib [172] have been shown to enhance bortezomib-induced apoptosis. Additionally, Akt activation is regulated by receptor tyrosine kinase growth factor receptors like EGFR, and Akt activation can be reversed with selective RTK inhibitors in these cells [175], leading to increased bortezomib sensitivity [176, 177].

#### 1.4.2.5 Overexpression of Other Growth-Related Proteins

Resistance to bortezomib has also been attributed to the overexpression of some proteins that are involved in cell growth, including interleukin-6 (IL-6) and insulin-like growth factor 1 (IGF-1), which are thought to confer resistance via activation of NF- $\kappa$ B through the PI3K/Akt and Raf/MEK/ERK pathways [178, 179]. IL-6 has been shown to play an important role in regulating drug sensitivity in multiple myeloma cells through inhibition of miRNA expression in bone marrow stromal cells (BMSCs) [180, 181]. In addition, IGF-1 receptor levels have also been shown to be high in multiple myeloma, and this overexpression, as well as increased IGF-1 levels, are associated with disease progression and poor patient prognosis [182, 183].

Increased IGF-1 signaling has been directly implicated in the resistance phenotype of bortezomib-resistant multiple myeloma cells with no  $\beta 5$  mutations. The role of IGF-1 signaling was further validated by gene expression profiling which showed that genes activated by IGF-1 were constitutively expressed in these bortezomib-resistant multiple myeloma cells. Importantly, blocking PI3K and mTOR downstream of IGF-1 partially overcame the bortezomib resistance. Direct inhibition of IGF-1R (insulin-like growth factor 1 receptor) was also able to sensitize cultured cells, in vivo models, and patient samples to bortezomib treatment [184], suggesting that combining bortezomib with IGF-1R inhibitors may be a promising strategy to prevent or overcome proteasome inhibitor resistance.

The receptor tyrosine kinase c-Met is also overexpressed in human myeloma cell lines and has been shown to promote drug resistance. One study showed that knock-down of c-Met in U266 human multiple myeloma cells enhanced their sensitivity to bortezomib via inhibition of the Akt/mTOR pathway [185]. Increased Akt/mTOR phosphorylation was also reported in bortezomib-resistant mantle cell lymphoma cells, and dual inhibition of PI3K and mTOR overcame acquired bortezomib resistance by suppressing the activated Akt/mTOR pathway [186]. Microarray analysis has also identified Rad (Ras associated with diabetes) as a potential factor in proteasome inhibitor resistance. Rad levels were increased in bortezomib-resistant Jurkat-R cells compared to parental controls, and knockdown resulted in induction of the mitochondrial apoptotic pathway via Noxa/Bcl-2, thus overcoming bortezomib resistance in these cells [187].

#### 1.4.2.6 Altered Autophagy Pathways

Proteasome inhibitors are known to activate autophagy, but the exact role of autophagy in cancer cell death is a controversial one [188]. Studies have reported that inhibition of autophagy can both inhibit [189] and promote [190] proteasome inhibitor-mediated cell death depending on cell type. This may be due to the variable effects of these autophagy inhibitors, whereby they block macroautophagy but are unable to inhibit chaperone-mediated autophagy, which may play a critical role in clearing protein aggregates in some cells. These protein aggregates may be transferred to the lysosome via aggresomes during chaperone-mediated autophagy. HDAC6 is necessary for aggresome formation following proteasome inhibition, and HDAC inhibition has been shown to enhance proteasome inhibitor-induced cell death in proteasome inhibitor-sensitive cells and to reverse resistance in resistant cells [191, 192]. The combination of HDAC inhibition with proteasome inhibition has been extensively studied, and results suggest that this is the most promising combination. In fact, one phase I clinical trial investigating the combination of bortezomib and the pan-HDAC inhibitor SAHA was completed in patients with relapsed/refractory multiple myeloma [193], and another was completed in patients with solid tumors [NCT00310024; National Cancer Institute]. A phase II trial also investigated the combination in patients with progressive, recurrent glioblastoma [NCT00641706; National Cancer Institute], and results are forthcoming.

### 1.4.2.7 Increased Antioxidants

The production of ROS appears to play a role in cell death induced by some proteasome inhibitors, which suggests that antioxidant protection mechanisms may also contribute to proteasome inhibitor resistance. Sensitivity of multiple myeloma cells has been shown to increase following depletion of intracellular reduced glutathione by buthionine sulfoximine treatment [194]. Glutathione may promote resistance by acting as a cofactor for GSH-dependent enzymes; protein disulfide isomerase, glutathione peroxidase, and vitamin C, for example, inhibit toxicity induced by proteasome inhibitors, among other factors [195–199]. Thus, antioxidant levels may impact proteasome inhibitor sensitivity, so regulating these levels may be a strategy to overcome resistance.

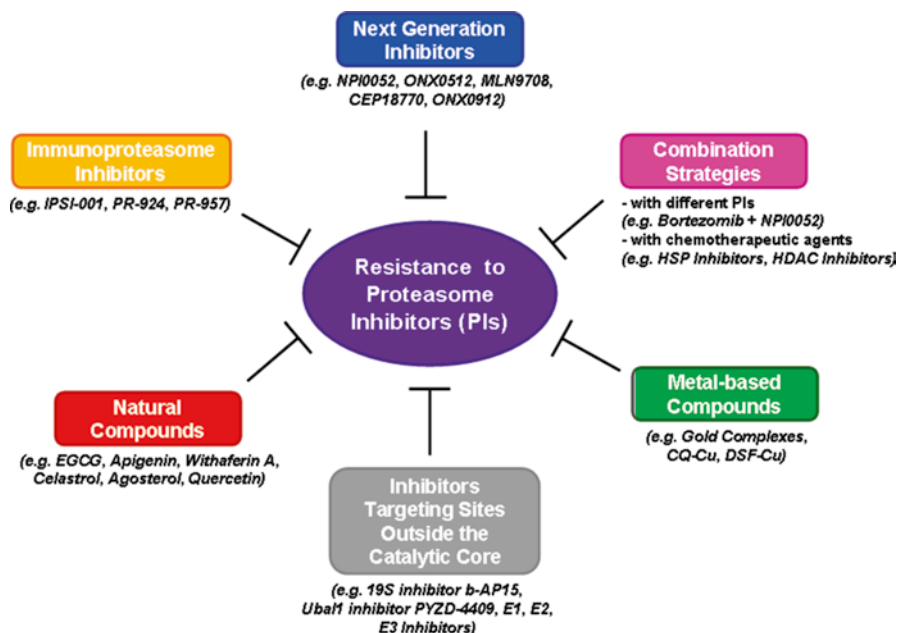
## 1.5 Measures to Overcome Proteasome Inhibitor Resistance

### 1.5.1 Design of Novel Proteasome Inhibitors

Determination of exact molecular mechanisms of proteasome inhibitor resistance would help in the design of effective therapeutic strategies to overcome proteasome inhibitor resistance. Resistance at the proteasome level could be addressed by designing better, more potent inhibitors than bortezomib and carfilzomib. A new generation of irreversible proteasome inhibitors might be helpful in partially overcoming bortezomib resistance due to  $\beta 5$  overexpression. Targeting sites different than those targeted by bortezomib could also be explored for the design and development of next-generation proteasome inhibitors. Unfortunately, however, these next-generation inhibitors may not be effective in overcoming resistance due to downstream factors [133].

Bortezomib is administered intravenously and modification of its pharmacokinetic parameters affecting stability, metabolism, and tissue bioavailability may be a useful strategy for overcoming resistance [133]. Some orally bioavailable reversible second-generation inhibitors, like MLN9708 and CEP 18770, that can be hydrolyzed to an active form have been developed, and encouraging results in cultured cells and animal models have advanced these compounds to phase I clinical trials [200–202]. However, their similarities to bortezomib in terms of mode of action might hinder their ability to overcome bortezomib/carfilzomib resistance, but they may offer advantages in terms of pharmacokinetics and patient compliance due to their oral route of administration, dosing flexibility and convenience [133].

Structure–activity relationship (SAR) studies were conducted to develop orally bioavailable carfilzomib-like agents, and led to the discovery and development of ONX0912, a truncated carfilzomib analog with comparable potency, selectivity, and anticancer activities to its parent compound in vitro and in vivo in animal models [203, 204]. Carfilzomib and ONX0912 can be degraded by proteases and peptidases in the plasma due to their peptide-like structures, which decreases their half-life and efficacy [133]. Therefore, nonpeptidic, irreversible proteasome



**Fig. 1.4** Potential strategies to overcome proteasome inhibitor resistance. Many strategies have been suggested for overcoming proteasome inhibitor resistance. These include designing novel proteasome inhibitors, targeting sites outside the catalytic center (such as the 19S regulatory cap, E1, E2s, or E3s), targeting the immunoproteasome, combination strategies (like proteasome inhibitors + HSP or HDAC inhibitors), and using metal-based or natural compounds as inhibitors

inhibitors like NPI0052 (salinosporamide/marizomib) with better bioavailability have been developed [205]. NPI0052 is a  $\beta$ -lactone- $\gamma$ -lactam isolated from the marine bacterium *Salinispora tropica* that shows potent irreversible inhibition of all three proteolytic activities of the proteasome through the formation of very stable acyl-ester bonds. Due to its anticancer activities in cell culture and animal models, NPI0052 has advanced into clinical trials for hematological and solid tumor malignancies (Fig. 1.4) [116, 206]. Larger in vitro studies should be conducted to further enhance the understanding of mechanisms associated with resistance to proteasome inhibitors. These studies could lead to the development of personalized therapeutic approaches by identifying subgroups of patients who are more likely to respond well or fail to respond to particular proteasome inhibitors.

### 1.5.2 Combination Strategies

In addition to designing new compounds to target the proteasome, combining current proteasome inhibitors with distinct modes of action could be an effective strategy to overcome resistance to particular proteasome inhibitors. For instance, NPI0052 in combination with bortezomib has been shown to induce synergistic

cytotoxicity *in vitro* in cultured multiple myeloma cells as well as in multiple myeloma cells isolated from patients, and in multiple myeloma mouse models (Fig. 1.4) [207]. Alternatively, proteasome inhibitors could be combined with other chemotherapeutic agents targeting factors downstream of the proteasome to enhance efficacy. In fact, lenalidomide and NPI0052 in combination display synergistic anti-multiple myeloma activities in cultured and patient multiple myeloma cells, as well as in tumor xenografts in mice [208].

Because HSP overexpression has been suggested as a potential mechanism by which cells become resistant to proteasome inhibitors, combining HSP inhibitors with proteasome inhibitors may sensitize resistant cells to proteasome inhibition (Fig. 1.4). In fact, the combination of the HSP-90 inhibitor tanespimycin (17-allylamino-17-demethoxy-geldanamycin [17-AAG]; geldanamycin analog) with bortezomib resulted in bortezomib-mediated cell death and tumor regression in multiple myeloma cell and xenograft models, respectively [148]. A phase I trial combining these drugs has also been completed in relapsed/refractory multiple myeloma patients, some of whom had progressed to bortezomib resistance [209]. An overall response rate of 27 % was achieved, suggesting that this combination is effective at reversing bortezomib resistance in multiple myeloma.

As discussed previously, some HDACs have also been implicated in proteasome inhibitor resistance, indicating that combining HDAC inhibitors with proteasome inhibitors may be a promising strategy to overcome proteasome inhibitor resistance (Fig. 1.4). Several preclinical studies have reported synergism between these types of drugs. For example, knockdown of HDAC1 enhanced bortezomib-mediated apoptosis, while overexpression of HDAC1 resulted in bortezomib resistance in multiple myeloma cells and treatment with the HDAC inhibitor romidepsin restored bortezomib sensitivity in HDAC1 overexpressing cells and tumor xenografts [210]. Additionally, the combination of bortezomib and tubacin (HDAC6-specific inhibitor) inhibited proliferation in cultured multiple myeloma cells [211]. Selective inhibition of HDAC6 by ACY-1215, both alone and in combination with bortezomib, has also been shown to suppress multiple myeloma cell growth in a xenograft mouse model, again suggesting a synergistic relationship [212]. Finally, the combination of bortezomib and the HDAC inhibitor SAHA (vorinostat) has also been evaluated in a phase I clinical trial in patients with advanced multiple myeloma, the results of which reported an overall response rate of 30 % in bortezomib-resistant patients [193]. Another phase I trial investigating the efficacy of SAHA in combination with bortezomib resulted in one partial remission and one minimal response among three bortezomib-resistant patients [213]. Another trial in multiple myeloma patients combined the natural HDAC inhibitor romidepsin with bortezomib, and 60 % partial and 8 % complete responses were achieved [214]. The Vantage 095 phase IIb trial reported an overall response rate of 18 % with a median duration of response of 6.3 months in patients with bortezomib-refractory relapsed multiple myeloma [215]. A randomized, double-blind phase III trial, Vantage 088, compared SAHA or placebo in combination with bortezomib in 637 myeloma patients and reported an overall response rate of 54 % in the group treated with the SAHA–bortezomib combination, compared with 41 % in the placebo group [216]. Thus, HDAC inhibition may also be a promising strategy for overcoming proteasome inhibitor resistance in refractory cancers.

### 1.5.3 Immunoproteasome-Specific Inhibitors

The immunoproteasome is an inducible proteasome variant primarily expressed in lymphocytes and monocytes, as well as in cells exposed to inflammatory cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). The immunoproteasome is involved in the production of peptides for major histocompatibility complex-1 (MHC-1). During assembly of the immunoproteasome, the constitutive  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 subunits are replaced by  $\beta$ 1i (PSMB9/LMP2),  $\beta$ 2i (PSMB10/LMP-10/MECL1), and  $\beta$ 5i (PSMB8/LMP7), respectively. These are associated with the 11S peptidase regulator and the remaining subunits of the constitutive proteasome, which leads to alterations in substrate specificity [217, 218]. Although there may be some functional redundancy between the two proteasome isoforms [219–221], CT-like ( $\beta$ 5i) and trypsin-like ( $\beta$ 2i) proteolytic activities are upregulated, and PGPH-like activity ( $\beta$ 1i) is decreased in the immuno-20S compared to the constitutive 20S [222]. Many conventional proteasome inhibitors (e.g., bortezomib, carfilzomib, NPI0052) designed to target the constitutive proteasome are also able to inhibit the immunoproteasome and thus offer broader activity at the cost of specificity, which may contribute to adverse effects [223].

Therefore, specifically targeting the immunoproteasome in hematological malignancies might be a novel approach toward increasing effectiveness and reducing negative off-target effects (Fig. 1.4) [223, 224], which ultimately led to the development of IPSIs. One such agent, IPSI-001, has shown selectivity for the immunoproteasome over the constitutive proteasome in binding assays and has been shown to induce apoptosis in a dose-dependent manner in patient derived cells of hematologic malignancies. IPSI-001 was also able to overcome resistance to conventional chemotherapeutic agents like doxorubicin, melphalan and, most importantly, bortezomib in vitro [224]. Other agents like the  $\beta$ 5i selective tripeptide epoxyketone-based immunoproteasome inhibitors PR-924 [225] and PR-957 [226] have also shown promising results in preclinical cell culture and animal studies. Synthetic analogs of the epoxyketone dihydroeponemycin were also developed as molecular probes to study the effects of  $\beta$ 1i (LMP2) inhibition. The combination of  $\beta$ 1i inhibitors with the  $\beta$ 5i inhibitor lactacystin resulted in enhanced inhibition of total CT-like activity compared to each agent alone. These inhibitors have also shown growth inhibitory effects in PC-3 prostate cancer cells overexpressing  $\beta$ 1i [227]. Taken together, these results indicate that targeting the immunoproteasome may be an effective strategy for overcoming resistance to conventional proteasome inhibitors.

### 1.5.4 Targeting Sites Other than the Catalytic Center

#### 1.5.4.1 E1, E2s, and E3s

Targeting other factors in the UPP may also prove effective in overcoming resistance associated with inhibitors of the 20S catalytic core (Fig. 1.4). Although inhibiting the ubiquitin E1 enzyme was initially disregarded due to potential lethality, the

identification of two natural E1 inhibitors, panepophenanthrin and himeic acid, has suggested that this may indeed be a viable strategy. Both inhibitors specifically inhibit the formation of E1 ubiquitin thioester intermediates [228, 229]. Additionally, PYR-41, a synthetic pyrazone derivative, with E1 inhibitory activity that prevents protein degradation and cytokine-mediated activation of NF- $\kappa$ B has also been developed [230]. Another compound, PYZD-4409 induced cell death in malignant cells as well as in a leukemia mouse model, potentially by a mechanism similar to ER stress induced by proteasome inhibitors [231]. Similarly, cell-based screening identified NSC624206 as an E1 inhibitor, though more studies are necessary to determine its molecular effects [232]. Following the observation that functional knockdown of the E2 Ubc13 results in increased p53 activity [233], inhibition of the E2 enzymes has also been explored. In fact, leucettamol A, a natural compound, has been reported to inhibit the interaction between the E2 Ubc13 and the inactive conjugating enzyme variant Uev1A, which is required for efficient poly-ubiquitin chain formation [234].

Perhaps one of the most widely researched strategies for targeting factors upstream of the proteasomal catalytic core is inhibition of ubiquitin E3 ligases (Fig. 1.4), likely due to their role in identifying target proteins for ubiquitination. E3 ligases are divided into one of three classes, RING, HECT, and U-box, based on domain structure and mechanisms of target recognition. The p53-specific RING-type E3 MDM2/HDM2 is a popular target for inhibition, due to its high frequency of overexpression in human cancers [235]. Indeed, nutlin-3, a MDM2 small molecule inhibitor, has been shown to suppress tumor progression in mouse xenograft models [236], suggesting that MDM2 is a promising target. Additionally, in bortezomib-sensitive multiple myeloma and epithelial carcinoma cells, nutlin-3 in combination with bortezomib resulted in additive and synergistic cytotoxic effects, respectively [237]. Some natural products, including chlorofusin and (-)-hexylitaconic acid, that inhibit the interaction between MDM2 and p53 have also been identified [238–241]. Interestingly, disulfiram and its derivatives have also been investigated for their ability to inhibit zinc finger- and RING-finger-containing ubiquitin E3 ligases [242]. Thus, inhibition of upstream UPP factors should be further investigated as this may be a viable strategy for overcoming resistance to 20S inhibitors.

#### 1.5.4.2 19S Regulatory Subunit

Inhibition of proteasomal regulators may also be effective in overcoming resistance to conventional proteasome inhibitors, as this inhibition should only hinder some proteasomal functions (Fig. 1.4). Indeed, screening of a library of purine analog-capped peptoids identified RIP-1 (regulatory particle inhibitor peptoid-1) as an inhibitor of protein unfolding through targeting of the ATPase Rpt4 [243, 244]. Reports have indicated that ubistatin A is capable of blocking recruitment of ubiquitinated proteins to the 26S proteasome by binding ubiquitin chains, ultimately suppressing proteasome-mediated proteolysis [245], indicating that ubiquitin chain receptors may also be good drug targets. Inhibition of deubiquitinase activity of the regulatory particle could be another useful strategy, and b-AP15, a small molecular

weight compound that inhibits deubiquitinating enzymes like USP14 and UCHL5, but not POH1, has shown anticancer activity in solid tumor models [246]. Thus, it is clear that factors regulating the 20S catalytic core are good drug targets, and further investigation into this strategy as a way to overcome inhibitors of the 20S core would be very worthwhile.

## 1.6 Nontraditional Options Targeting the 20S Core

### 1.6.1 *Metals in Cancer Development and Therapy*

Just as proteasome activity levels have been shown to be altered in cancer, so have levels of various metals like copper [247–252] and zinc [253–256], a discovery which has led to extensive research regarding the roles of these metals in the development of human cancers as well as their potential as anticancer therapeutics.

The discovery that some metal-based compounds, like cisplatin, possess potent anticancer properties, coupled with the importance of copper and zinc to essential biological processes like tumorigenesis, has led to the investigation into copper and zinc as metal centers in anticancer drugs. Since its discovery over four decades ago, cisplatin has cured over 90 % of testicular cancer cases, and it has also played a critical role in the treatment of various other cancers, including lymphoma, melanoma, bladder, cervical, and ovarian [257]. Unfortunately, although cisplatin use has proven effective, it has also been associated with toxicity and resistance, which has limited its use [258, 259] and prompted the search for less toxic metal-based drugs, including second-generation platinum drugs, as well as complexes containing metals like cobalt, copper, gallium, gold, tin, and zinc, among others.

### 1.6.2 *Metal-Based Complexes as Proteasome Inhibitors*

#### 1.6.2.1 *Gold-Containing Complexes*

Because of the successful use of gold compounds in other diseases [260, 261], gold compounds have also been investigated for their potential anticancer activity (Fig. 1.4). Gold (I) complexes, including auranofin analogs, exhibited potent cytotoxic activity against B16 melanoma and P388 leukemia cells [262], but phosphine-gold(I) thiosugars were the most potent, and while active against leukemia in vivo, these analogs were completely inactive in solid tumor models [263]. Gold(III) complexes have also been investigated, in spite of initial trepidation due to their high redox activity and poor stability. Au(III) is expected to be reduced to Au(I) and metallic Au in the reducing tumor microenvironment, potentially making Au(III) complexes less effective [264]. Various Au(III) compounds with ligand platforms containing nitrogen atoms as donor groups [265], exhibiting a superior



chemotherapeutic index, increased cytotoxicity, and fewer toxic side effects than cisplatin [264], were investigated for their antitumor abilities. One example is Au (DMDT) Br<sub>2</sub>, which significantly inhibited CT-like activity in purified 20S proteasome (IC<sub>50</sub>=7.4 μM) and 26S proteasome in intact MDA-MB-231 breast cancer cells (10–20 μM) and breast tumor xenografts [266]. Another gold(III) compound, AUL12, was also shown to exhibit potent proteasome-inhibitory and cell death-inducing activities in MDA-MB-231 breast cancer cells (IC<sub>50</sub>=4.5 μM, 70 % inhibition). Interestingly, treatment with this Au(III) compound was associated with redox processes, indicating that induction of oxidative stress may be partially responsible for the cytotoxicity of gold(III) compounds [267].

### 1.6.2.2 Metal Chelators as Proteasome Inhibitors

The success of metal-containing drugs, along with the functional importance of metals like copper and zinc to normal cellular function, has resulted in studies exploring chelation of these essential metals with chelators like dithiocarbamate and hydroxyquinolone compounds, several of which have been previously approved for the treatment of myriad diseases, such as AIDS, alcoholism, and bacterial and fungal infections [268–270].

#### Dithiocarbamates

Dithiocarbamate compounds, including disulfiram, are known to form metal complexes, a property that has been applied as a potential strategy to target the UPP in cancer. Disulfiram (tetraethylthiuram disulfide, DSF) is an irreversible aldehyde dehydrogenase inhibitor that is one of two drugs approved by the USFDA for the treatment of alcoholism [271–273]. When complexed with copper, disulfiram can potently inhibit both purified 20S (IC<sub>50</sub>=7.5 μM) and intact 26S proteasome in MDA-MB-231 breast cancer cell lysates (20 μM; >95 % inhibition), as well as inducing apoptosis in the cultured cells [274]. DSF alone, however, had no effect, which is unsurprising, since cultured cells do not express high levels of copper. Significant inhibition (74 %) of tumor growth was also observed in female athymic nude mice bearing MDA-MB-231 tumor xenografts, associated with an 87 % decrease in CT-like activity [274]. Together, the results indicate that the increased copper levels observed in human tumors may be exploited as an anticancer mechanism (Fig. 1.4).

The results of these and other preclinical studies of DSF have also led to a number of clinical trials investigating the use of DSF in humans. One phase I/II clinical trial evaluated the efficacy of DSF in stage IV metastatic melanoma patients [NCT00256230; UC-Irvine] and another examined the effects of DSF on PSA levels in recurrent prostate cancer patients [NCT01118741; Johns Hopkins University]. Two other trials investigated the effects of DSF in combination treatments. The first evaluated the toxicity profile and safety of coadministration of DSF and copper gluconate in refractory malignancies with liver metastases

[NCT00742911; Huntsman Cancer Institute], and the other determined the effects of addition of DSF to current chemotherapeutic treatments in patients with non-small cell lung cancer [NCT00312819; Hadassah Medical Organization]. All of these trials have been completed, but results are as yet unavailable.

## Hydroxyquinolones

Hydroxyquinolones are another class of metal-chelating compounds that have been investigated for their anticancer properties. One example is clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, CQ), a lipophilic compound that can form stable complexes with copper (II) [275]. CQ has been shown to reduce and prevent the formation of amyloid plaques in Alzheimer's disease transgenic mice [276], a discovery that led to two clinical trials that validated the efficacy of CQ in Alzheimer's disease with no visible toxicity [277, 278]. Consequently, CQ is currently used for the treatment of Alzheimer's and Huntington's diseases [279, 280]. A CQ–Cu complex (1:1 molar ratio) inhibited both purified 20S ( $IC_{50}=2.5\ \mu\text{M}$ ) and intact proteasome (20  $\mu\text{M}$ ) in LNCaP and C4-2B prostate cancer cells (82 % and 83 %, respectively). Additionally, mice bearing C4-2B xenografts treated with CQ exhibited significant tumor growth inhibition (66 %), as well as inhibition of angiogenesis and the proteasome and induction of apoptosis [281]. These data clearly indicate that compounds like DSF and CQ require copper to be transported into cancer cells in order to exert their proteasome-inhibitory and apoptosis-inducing abilities [282], but that when copper is present, they are quite potent proteasome inhibitors that are minimally toxic toward normal cells (Fig. 1.4) and therefore, they may be exploited as potential novel strategies for overcoming resistance to traditional proteasome inhibitors like bortezomib.

### 1.6.3 Natural Compounds as Proteasome Inhibitors

While much emphasis has been placed on the development of synthetic proteasome inhibitors to overcome resistance to proteasome inhibitors, the use of natural compounds and their analogs or derivatives might be a better strategy as many phytochemicals and marine products have shown proteasome-inhibitory and subsequent anticancer activities (Fig. 1.4) [283]. Some examples of natural products investigated for their potential as proteasome inhibitors include Withaferin A, celastrol, agosterols, green tea polyphenols, and apigenin. Withaferins are isolated from the medicinal plant “Indian winter cherry” or “Indian ginseng” (*Withania somnifera*), and have been widely used in traditional Indian “Ayurveda” medicine. Specifically, Withaferin A has been reported to possess anticancer abilities, which may be partly attributed to inhibition of CT-like activity [284]. Celastrol, a triterpene isolated from the Chinese “Thunder of God Vine” (*Tripterygium wilfordii*) has also shown proteasome-inhibitory activity leading to the accumulation of ubiquitinated proteins

and proteasomal target proteins in both androgen receptor positive and negative prostate cancer cell lines [285]. Agosterols, isolated from the marine sponge *Acanthodendrilla* sp., have shown inhibition of CT-like activity in rat proteasome in the low micromolar range and also induced cytotoxicity in HeLa cervical cancer cells [286]. The proteasome-inhibitory, apoptosis-inducing activities of green tea polyphenols and apigenin have been more extensively studied, with both advancing to clinical trials.

### 1.6.3.1 Green Tea Polyphenols

Tea, derived from the *Camellia sinensis* plant, is the most popular beverage in the world after water. Tea comes in many varieties, including green, black, and oolong, all of which contain many beneficial compounds. The most potent of these are polyphenols, which are characterized by the presence of more than one phenol group per molecule and are believed to provide the coloring in many plants [287].

The most active polyphenol in tea is (-)-epigallocatechin-3-gallate, or (-)-EGCG, which has been shown to possess anticancer activity in several cancer types, including bladder, breast, and B-cell malignancies (Fig. 1.4) [288–290]. The proteasome-inhibitory activity of (-)-EGCG has been explored *in vitro* and *in vivo*. (-)-EGCG inhibited CT-like activity in both purified 20S ( $IC_{50}=86\text{--}194$  nM) and intact 26S (from Jurkat leukemia, LNCaP and PC-3 prostate cancer, and MCF-7 breast cancer cell extracts) proteasome, and increased ubiquitinated proteins, p27, and I $\kappa$ B- $\alpha$  were also observed [291]. Unfortunately, however, a recent study using experimental multiple myeloma models has revealed a direct interaction between (-)-EGCG and bortezomib that inhibits the efficacy of bortezomib [292], but whether green tea consumption affects the efficacy of bortezomib therapy in multiple myeloma patients needs to be confirmed.

The interesting preclinical data, coupled with the popularity of tea, have led to clinical trials using (-)-EGCG and other green tea polyphenols. One phase I trial evaluated the optimal dose and tolerability of (-)-EGCG in previously untreated, asymptomatic chronic lymphocytic leukemia patients, and results indicated that (-)-EGCG is tolerable and does result in some clinical benefit [293]. Another phase I study investigated the effects of (-)-EGCG supplementation on serum levels of prostate cancer biomarkers [294], with all prostate cancer-associated biomarkers, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, IGF-binding protein-3 (IGFBP-3), and prostate-specific antigen (PSA) decreasing significantly after treatment with no elevation of liver function enzymes. Therefore, even short-term (-)-EGCG treatment may be clinically beneficial to prostate cancer patients [294].

In addition, a number of phase I and II trials examining the effects of (-)-EGCG treatment in various types of cancer, including nonmetastatic bladder, breast, cervical, colorectal, prostate, non-small cell lung, and uterine carcinomas as well as multiple myeloma, are currently recruiting patients or are ongoing. Other studies are investigating the potential preventive effects of (-)-EGCG in patients at risk for

cervical, esophageal, and lung cancers. Finally, still others are evaluating (-)-EGCG in combination with the EGFR inhibitor erlotinib in non-small cell lung cancer and premalignant lesions of the head and neck. The preclinical and clinical data, as well as the numerous recruiting/ongoing clinical trials, clearly support the use of (-)-EGCG as a chemopreventive or therapeutic agent, and its potential use following progression to resistance to other proteasome inhibitors should be explored.

### 1.6.3.2 Apigenin

Apigenin (5,7,4-trihydroxyflavone) has also been shown to possess antioxidant, antimutagenic, and chemopreventive properties (Fig. 1.4). Apigenin is a dietary flavonoid found in various natural products including celery seed, chamomile flowers, grapes, and parsley [295–298], and although the mechanism is not fully understood, chemoprevention by apigenin has been reported in several cancers including cervical [299], lung [300], prostate [301], and skin [302].

Studies have shown that the carbonyl carbon in the C4 position of apigenin binds to the  $\beta 5$  subunit in a suitable orientation for nucleophilic attack by the N-terminal Thr1 [303]. Apigenin potently inhibits CT-like activity of purified 20S ( $IC_{50}$  = 1.8–2.3  $\mu$ M) and intact proteasome in Jurkat leukemia cell lysates (1–10  $\mu$ M), with little to no toxicity in immortalized, non-transformed natural killer cells [303]. Proteasome inhibition-associated accumulation of ubiquitinated proteins and apoptosis-associated morphological changes, activation of caspase-3/-7, and cleavage of PARP were also observed, and similar proteasome inhibition and apoptosis induction were observed in breast cancer MBA-MD-231 cells and tumors, with no significant changes in body weight following apigenin treatment, indicating low toxicity in vivo [304]. The promising preclinical data have led to examination of the efficacy of apigenin in human patients. In one prospective study in patients with resected colon cancer or who had undergone polypectomy, one group received a flavonoid mixture and the other served as a matched control. The results suggested that dietary consumption of flavonoids like apigenin may reduce the risk of colorectal cancer [305]. This preventive effect has not been fully validated, however, and further studies are necessary to determine if apigenin could be used to overcome resistance to other proteasome inhibitors.

## 1.7 Conclusion

The clinical approval of the proteasome inhibitors bortezomib and carfilzomib was validation of the importance of the UPP as a critical anticancer therapeutic target. Unfortunately, intrinsic and acquired resistance in tumor cells is associated with the use of clinical proteasome inhibitors, so it is important to find novel strategies to overcome this resistance. Resistance may be due to a variety of factors including overexpression or mutation of the  $\beta 5$  subunit, overexpression of HSPs, altered

expression of apoptosis- and growth-related proteins, Akt pathway activation, altered autophagy, and increased antioxidant levels. Toward the goal of overcoming this resistance, novel small molecules have been tested for their ability to selectively target and inhibit components of the UPP other than the catalytic 20S core. These novel targets include the 19S regulatory cap(s), deubiquitinating enzymes, and the enzymes involved in the ubiquitination cascade (E1, E2s, and E3s). Inhibitors containing metal centers and those derived from natural products may also be viable options for overcoming resistance associated with clinical proteasome inhibitors. Finally, targeting the specialized immunoproteasome also has potential as a valuable new strategy that may be more specific and could overcome resistance to constitutive 20S proteasome inhibitors. Therefore, the UPP is a promising target for cancer therapy, but further studies are necessary to develop inhibitors that can avoid the resistance associated with clinical proteasome inhibitors.

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