

# The ubiquitin–proteasome system: opportunities for therapeutic intervention in solid tumors

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## Abstract

The destruction of proteins via the ubiquitin–proteasome system is a multi-step, complex process involving polyubiquitination of substrate proteins, followed by proteolytic degradation by the macromolecular 26S proteasome complex. Inhibitors of the proteasome promote the accumulation of proteins that are deleterious to cell survival, and represent promising anti-cancer agents. In multiple myeloma and mantle cell lymphoma, treatment with the first-generation proteasome inhibitor, bortezomib, or the second-generation inhibitor, carfilzomib, has demonstrated significant therapeutic benefit in humans. This has prompted United States Food and Drug Administration (US FDA) approval of these agents and development of additional second-generation compounds with improved properties. There is considerable interest in extending the benefits of proteasome inhibitors to the treatment of solid tumor malignancies. Herein, we review progress that has been made in the preclinical development and clinical evaluation of different proteasome inhibitors in solid tumors. In addition, we describe several novel approaches that are currently being pursued for the treatment of solid tumors, including drug combinatorial strategies incorporating proteasome inhibitors and the targeting of components of the ubiquitin–proteasome system that are distinct from the 26S proteasome complex.

## Key Words

- ▶ proteasome
- ▶ bortezomib
- ▶ carfilzomib
- ▶ oprozomib
- ▶ deubiquitinase
- ▶ solid tumor

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## Introduction

The highly regulated degradation of cellular proteins is critically important for the ability of normal healthy cells to proliferate and differentiate (Ciechanover 2005). Similarly, induction of apoptosis in normal cells depends, in part, on selective protein degradation, leading to a decrease in the ratio of anti- vs pro-apoptotic proteins in the cell. The vast majority of protein degradation in the cell is accomplished via the ubiquitin–proteasome system, wherein proteins destined for degradation are covalently tagged with ubiquitin, and then subjected to proteolytic destruction by the macromolecular proteasome complex.

Cancer cells are commonly characterized by the over-expression or hyperactivation of proteins that promote aberrant progression through the cell cycle or suppression of apoptosis, or by the loss of proteins that are important for cell cycle checkpoints or the induction of apoptosis. A prevailing school of thought has suggested that inhibition of the proteasome may lead to the accumulation of proteins deleterious to the survival of cancer cells, allowing restoration of cell cycle arrest and/or apoptotic cell death. Indeed, numerous preclinical studies have now shown that inhibition of the proteasome results in

significant anti-cancer effects both *in vitro* and *in vivo* (Chen *et al.* 2011, Frankland-Searby & Bhaumik 2012).

Bortezomib is a first-in-class reversible inhibitor of the proteasome that has achieved considerable success in the treatment of certain hematologic malignancies. Notably, the United States Food and Drug Administration (US FDA) has approved the use of bortezomib for multiple myeloma and mantle cell lymphoma (Kane *et al.* 2003, 2007, Richardson *et al.* 2003, 2005, Bross *et al.* 2004, Fisher *et al.* 2006). However, several factors limit both the short- and long-term success of bortezomib. Bortezomib exhibits considerable off-target effects that contribute to a high rate of peripheral neuropathy in treated patients (Richardson *et al.* 2006, Orłowski *et al.* 2007, Cavaletti & Jakubowiak 2010, Corso *et al.* 2010). In addition, bortezomib is not orally bioavailable, and the reversible nature of this agent requires frequent intravenous delivery to maintain prolonged proteasome inhibition. Furthermore, many tumors exhibit inherent resistance to bortezomib, and most sensitive tumors eventually develop acquired resistance (Richardson *et al.* 2003, 2005, 2006, Lonial *et al.* 2005, O'Connor *et al.* 2005, Orłowski *et al.* 2007). In an effort to improve on the success of bortezomib and to overcome some of the limitations associated with this agent, considerable effort has been invested in the identification and development of next generation proteasome inhibitors, including MLN9708 (Kupperman *et al.* 2010, Chauhan *et al.* 2011), carfilzomib (Demo *et al.* 2007, Kuhn *et al.* 2007), oprozomib (Zhou *et al.* 2009, Chauhan *et al.* 2010), marizomib (NPI-0052 or salinosporamide A; Feling *et al.* 2003, Chauhan *et al.* 2005, Macherla *et al.* 2005), and delanzomib (CEP-18870; Dorsey *et al.* 2008, Piva *et al.* 2008). All of these inhibitors are currently undergoing clinical evaluation in hematologic and/or solid tumor malignancies.

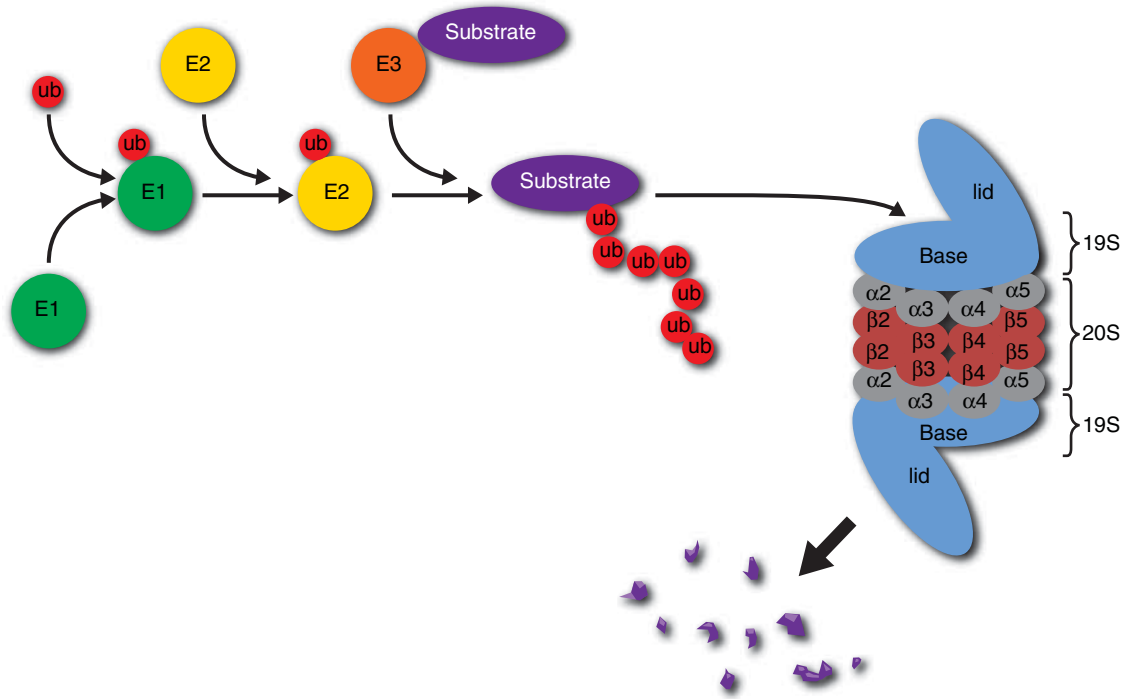
Despite the major impact that bortezomib treatment has had on multiple myeloma and mantle cell lymphoma therapies, considerably less success has been seen in solid tumors. There are probably a number of factors that contribute to this paucity of success, but chief among them appears to be the inherent resistance of solid tumors in *in vivo* settings. It is hoped that second-generation proteasome inhibitors with different selectivities for proteasome subunits, enhanced or prolonged potencies, or reduced side effects will generate more satisfying effects on solid tumors. Moreover, it appears likely that the anti-cancer activities of proteasome inhibitors will be markedly improved through the development of rational drug combination strategies incorporating conventional or molecular targeting agents. Lastly, the

ubiquitin–proteasome system is highly complex, involving regulatory and catalytic proteins beyond the central proteasome core. Efforts to target distinct components within this system are underway, and may provide a more efficacious way to convert highly proliferative or apoptosis-resistant solid tumor cells to a more vulnerable state. This review will focus on the basic steps and components of the ubiquitin–proteasome system, key proteins that are regulated by this system, the development and evaluation of small molecules targeting different system components, and the potential for combinatorial strategies against solid tumors.

### Protein degradation via the ubiquitin–proteasome system

Proteins destined for degradation via the ubiquitin–proteasome system include proteins that are damaged, improperly folded, or those that are intended to have short half-lives in the cell (Ciechanover 2005). Degradation of proteins by the ubiquitin–proteasome system is accomplished in two major steps: i) polyubiquitination of the protein and ii) proteolytic degradation of the polyubiquitinated protein by the macromolecular proteasome complex (Orłowski & Wilk 2000, Ciechanover 2005, Shen *et al.* 2013). Each of these steps involves a complex series of protein interactions and biochemical events (Fig. 1).

Polyubiquitination of substrate proteins first involves activation of the 76-amino acid ubiquitin polypeptide by the activating enzyme E1. In humans, one primary E1 enzyme (Ube1) has been identified, although it remains possible that others may be found. Activation involves covalent linkage between the carboxyl-terminus of ubiquitin and a cysteine residue present on E1, forming a thioester bond. The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme forming again a thioester covalent linkage. At least 50 distinct E2 enzymes have been identified in humans. In a third step, an E3 ligase enzyme transfers the ubiquitin from E2 to the substrate protein. As E3 proteins act to recognize and bind substrate proteins, it is not surprising that over 500 E3 enzymes appear to be encoded by the human genome. The majority of E3 ligases are classified as RING finger E3s, and act by bringing substrates and E2 enzymes into close proximity. The RING finger E3s then directly transfer ubiquitin from E2 to the substrate, without forming an intermediate covalent bond. A minority of E3 ligases (roughly 30) are classified as HECT domain E3s, and act by forming an intermediate thioester linkage with

**Figure 1**

Degradation of proteins via the ubiquitin–proteasome system. The degradation of a substrate protein via the ubiquitin–proteasome system involves polyubiquitination of the protein, followed by proteasomal degradation. Ubiquitination involves the activation of ubiquitin by E1, intermediate conjugation to an E2 ubiquitin-conjugating enzyme, then transfer of the ubiquitin to the substrate by an E3 ubiquitin ligase enzyme. The process is then repeated to achieve polyubiquitination of the substrate

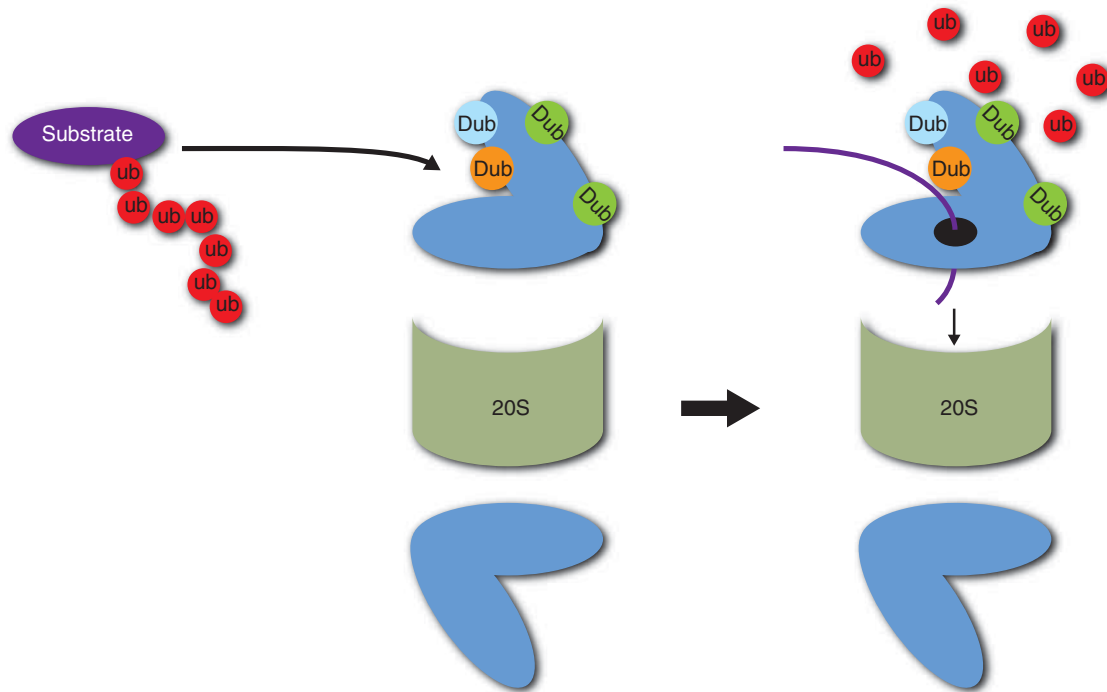
protein. The ubiquitinated substrate protein is then recognized for degradation by the 26S proteasome complex comprising two 19S regulatory particles and a 20S catalytic core particle. The caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (CT-L) activities of the 20S particle are present in the  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$  subunits respectively. Substrate proteins are degraded to oligopeptides.

ubiquitin before transfer to the substrate. The E3 enzymes ligate ubiquitin to lysine residues present on the substrate protein. Following monoubiquitination of the substrate, the process must be repeated to form an elongated chain of ubiquitin residues. Proper recognition of ubiquitinated substrates by the proteasome complex is thought to require a minimum of four ubiquitin residues in the polyubiquitin chain.

Proteins that have been appropriately polyubiquitinated are recognized and degraded by the 26S macromolecular proteasome complex (Gallastegui & Groll 2010). The 26S complex consists of a 20S catalytic core particle that is capped at both ends by 19S regulatory particles. The 19S regulatory particle can be further subdivided into lid and base components. Following recruitment to the proteasome, polyubiquitinated proteins undergo deubiquitination and unfolding (Fig. 2). The removal of ubiquitin is accomplished by a family of deubiquitinase (Dub) enzymes, some of which are associated with the 19S lid. Ubiquitin polypeptides

that are removed from substrate proteins can be directly recycled by the cell. The 19S base component plays a key role in unfolding of the substrate protein and delivery of the unfolded, deubiquitinated substrate into the 20S catalytic core particle. The 20S catalytic core particle consists of four layers of ring-like structures (Groll *et al.* 1997). The outer ring layers are composed of seven ‘alpha’ subunits,  $\alpha 1$ – $\alpha 7$ , while the inner ‘beta’ rings are composed of seven beta subunits,  $\beta 1$ – $\beta 7$ . The  $\beta 1$  subunits exhibit caspase-like (C-L) proteolytic activity, the  $\beta 2$  subunits exhibit trypsin-like (T-L) activity, and the  $\beta 5$  subunits exhibit chymotrypsin-like (CT-L) activity. Collectively, these subunits act to degrade substrate proteins into short oligopeptides.

In addition to the widely distributed, constitutive proteasome complex described above, cells in the immune system express an inducible form of the proteasome called the immunoproteasome (Basler *et al.* 2013). The immunoproteasome differs in the composition of the beta subunits and the regulatory particle. Treatment with

**Figure 2**

Substrate protein entry to the 20S catalytic core. Before entry into, and degradation by, the 20S catalytic core, polyubiquitinated proteins undergo deubiquitination by the members of the deubiquitinase (Dub) enzyme family. The released ubiquitin moieties are recycled by the cell.

The substrate protein also undergoes denaturation/unfolding. Entry of the substrate protein into the 20S catalytic core particle is regulated by the base component of the 19S regulatory particle.

interferon  $\gamma$  or tumor necrosis factor  $\alpha$  induces the expression of unique  $\beta 1i$  (LMP2),  $\beta 2i$  (MECL-1/LMP10), and  $\beta 5i$  (LMP7) subunits that replace the  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$  subunits of the constitutive proteasome. In addition, a unique 11S regulatory particle is induced, which replaces the 19S regulatory particle and caps the immunoproteasome complex. With elevated T-L and CT-L, and reduced C-L, activities, the immunoproteasome plays a key role in the generation of antigenic peptides that are used to generate MHC class I-mediated immune responses.

## Key proteins regulated by the ubiquitin–proteasome system

### p53

The tumor suppressor protein p53 acts as a transcription activator and plays key roles in the promotion of cell cycle arrest, as well as induction of apoptosis. Cellular levels of p53 are tightly regulated by the ubiquitin–proteasome system. HDM2, a RING finger E3 ligase, in complex with p300/CBP acts to promote polyubiquitination and rapid proteasomal degradation of the p53 protein. In view of the

capacity of p53 to promote either cell cycle arrest or apoptosis, inhibition of the interaction between p53 and HDM2, or inhibition of proteasome activity, as a means of elevating p53 levels in cancer cells has been intensively investigated (Brown *et al.* 2009). Of course, such a strategy requires that the cancer cells retain the capacity to express WT p53. However, the *TP53* gene is among the most commonly mutated or deleted gene in human cancers, limiting the applicability of this approach.

A unique situation, and opportunity, exists in cancers characterized by infection with human papilloma virus (HPV). Nearly, all cases of cervical carcinoma and an increasing number of head and neck cancers harbor HPV (Shiboski *et al.* 2005, Chaturvedi *et al.* 2008, 2011, Nasman *et al.* 2009). In HPV-associated cancer, the viral E6 protein, in concert with the E6-associated protein, acts as a HECT domain E3 ligase, promoting the ubiquitination and proteasomal degradation of p53 (Scheffner *et al.* 1993). As p53 is efficiently removed via an HPV E6-mediated process, HPV-positive cancer cells have little selective pressure to mutate or delete the *TP53* gene. Accordingly, nearly all cases of HPV-positive cervical carcinoma and head and neck cancer retain the ability to produce WT p53

(Balz *et al.* 2003, Poeta *et al.* 2007, Westra *et al.* 2008). This suggests that treatment with E6 or proteasome inhibitors may be particularly useful for restoring the expression of WT p53 in HPV-positive solid tumors.

### p27

The cyclin-dependent kinase (CDK) inhibitor p27 plays a key role in regulating the entry of quiescent cells into the cell cycle. Inhibition of the cell cycle by transforming growth factor  $\beta$  or cell–cell contact is mediated, in part, by p27, and proteasomal degradation of p27 allows resumption of cell cycle progression. Ubiquitination of p27 occurs via a complex series of biochemical events, ultimately involving the RING finger E3 ligase complex SCF<sup>Skp2</sup> (Shen *et al.* 2013). Interestingly, activation of the E3 ligase activity of the SCF<sup>Skp2</sup> complex requires the attachment of NEDD8, a polypeptide similar to ubiquitin, to a component of the protein complex (Merlet *et al.* 2009). As discussed later, this has spurred the development of neddylation inhibitors as a means of restoring p27 expression and cell cycle arrest in cancer cells.

### Cyclins

Cyclins associate with CDKs to promote CDK activation and drive progression through the cell cycle. More than 15 different cyclins have been identified in humans, each acting at a particular phase of the cell cycle. Cyclins were named due to the fact that their expression levels vary, or cycle, dramatically throughout the cell cycle. Cyclin levels are tightly regulated by both transcriptional induction and proteasomal degradation. Inhibition of the proteasome, resulting in aberrant expression of cyclins throughout the cell cycle, has the potential to promote inappropriate CDK activation and cell cycle progression. In cancer cells, this may have a therapeutic benefit by activating safeguard apoptosis or mitotic catastrophe cell death mechanisms.

### NOXA, BAX, and BIK

Multiple investigations have shown that inhibition of the proteasome leads to upregulation of pro-apoptotic members of the BCL2 protein family, including NOXA, BAX, and BIK (Qin *et al.* 2005, Zhu *et al.* 2005a,b, Fribley *et al.* 2006, Perez-Galan *et al.* 2007, Voortman *et al.* 2007a, Li *et al.* 2008). Moreover, the use of antisense oligonucleotides, siRNAs, or shRNAs, has shown that these family members are partially responsible for mediating cell death

induction by proteasome inhibitors in cell line models representing melanoma (Qin *et al.* 2005), head and neck cancers (Fribley *et al.* 2006, Li *et al.* 2008), and colon cancer (Zhu *et al.* 2005a). NOXA and BAX are known to be induced by p53 (Miyashita & Reed 1995, Oda *et al.* 2000), and their upregulation in response to proteasome inhibition may result from the elevation of p53 levels, although p53-independent mechanisms of NOXA and BAX upregulation have also been described (Qin *et al.* 2005). The mechanisms responsible for the potent upregulation of BIK that has been reported in proteasome inhibitor-treated solid tumor cell lines are less well understood.

### MCL1

In addition to causing upregulation of pro-apoptotic BCL2 family members, proteasome inhibitors have also been shown to dramatically increase the levels of MCL1, an anti-apoptotic BCL2 family member (Opferman 2006, Li *et al.* 2008, Zang *et al.* 2012a). Biochemical studies have shown that MCL1 is a direct proteasome substrate (Opferman 2006). Since MCL1 acts to inhibit apoptosis, co-treatment with a proteasome inhibitor and an inhibitor of MCL1 expression or function is likely to enhance the cell death-inducing activity of the proteasome inhibitor. Indeed, use of the MCL1 inhibitor, obatoclax (GX15-070) or shRNAs directed against *MCL1* mRNA, has been shown to markedly improve the potencies of proteasome inhibitors against cancer cells of hematologic or solid tumor origin (Perez-Galan *et al.* 2007, 2008, Li *et al.* 2008, Zang *et al.* 2012a).

### TRAIL receptors

The plasma membrane receptors, DR4 and DR5, bind and mediate the induction of the extrinsic apoptotic pathway by the death ligand TRAIL. Treatment of cells with proteasome inhibitors has been shown to upregulate the expression of DR4 and DR5, enhancing sensitivity to TRAIL (Nikrad *et al.* 2005, Liu *et al.* 2007, Voortman *et al.* 2007b, Shanker *et al.* 2008, Seki *et al.* 2010, Yoshida *et al.* 2011). Although the mechanism of DR4 and DR5 upregulation is incompletely understood, preclinical studies suggest the potential therapeutic benefit of co-treatment with TRAIL and proteasome inhibitors in solid tumors.

### NF- $\kappa$ B

The NF- $\kappa$ B transcription factor induces the expression of a wide variety of genes important for cellular proliferation

and survival, as well as inflammation and angiogenesis (Ghosh & Karin 2002). Normally, NF- $\kappa$ B is maintained in an inactive state through sequestration in the cytoplasm by the endogenous inhibitor I $\kappa$ B (Ghosh & Baltimore 1990). The activation of NF- $\kappa$ B by external stimuli involves phosphorylation of I $\kappa$ B, leading to I $\kappa$ B ubiquitination and proteasomal degradation (Traenckner *et al.* 1995). The liberated NF- $\kappa$ B migrates to the nucleus and therein promotes gene transcription. NF- $\kappa$ B is also well known to be constitutively overexpressed and/or hyperactivated in a wide variety of hematologic and solid tumor malignancies (Aggarwal 2004, Van Waes 2007). Treatment with proteasome inhibitors is a well-established approach for suppressing I $\kappa$ B degradation and, thereby, inhibiting the cancer-promoting activity of constitutively activated NF- $\kappa$ B (Traenckner *et al.* 1994).

## Inhibitors of the proteasome 20S catalytic subunit

### Bortezomib

Bortezomib (Millenium-Takeda Oncology) is the first proteasome inhibitor to be approved by the US FDA for the treatment of cancer (Kane *et al.* 2003, 2007, Richardson *et al.* 2003, 2005, Bross *et al.* 2004, Fisher *et al.* 2006). Bortezomib is a dipeptidyl boronate compound that binds to the  $\beta$ 5 subunit in a reversible fashion, inhibiting the CT-L activity of the 20S catalytic core particle (Chen *et al.* 2011). However, bortezomib is not entirely specific, with modest inhibitory activity against the  $\beta$ 1 subunit, as well as inhibitory activities against a variety of serine proteases, including cathepsins A and G, chymase, dipeptidyl peptidase II, and HtrA2/Omi (Arastu-Kapur *et al.*). It has been proposed that these nonspecific activities contribute to the high rate of peripheral neuropathy that has been observed in bortezomib-treated patients (Arastu-Kapur *et al.*). Owing to the reversible nature of bortezomib, prolonged inhibition of the proteasome *in vivo* may require relatively frequent administration, although this is somewhat mitigated by the slow rate of bortezomib dissociation from the  $\beta$ 5 subunit.

### MLN9708

MLN9708 (Millennium-Takeda Oncology) is an orally bioavailable analog of bortezomib currently undergoing Phase I/II clinical evaluation in hematologic and solid tumor malignancies (Kupperman *et al.* 2010, Chauhan *et al.* 2011, Driscoll & Woodle 2012). Like bortezomib,

MLN9708 is a peptide boronate compound. Following oral administration, the MLN9708 prodrug is metabolized to the active agent MLN2238, which acts as a reversible inhibitor of the  $\beta$ 5 subunit.

### Delanzomib

Delanzomib (Cephalon), also called CEP-18770, is an orally bioavailable peptide boronate that inhibits the  $\beta$ 5 subunit (Dorsey *et al.* 2008, Piva *et al.* 2008). Preclinical studies have indicated that delanzomib and bortezomib exhibit similar activities against hematologic and solid tumors. However, delanzomib may demonstrate reduced adverse toxicities (Piva *et al.* 2008). Specifically, in comparison with bortezomib, delanzomib has shown significantly reduced cytotoxicity toward bone marrow stromal cells, bone marrow progenitor cells, and normal human intestinal epithelial cells. Currently, delanzomib is undergoing clinical evaluation in Phase I/II trials ([clinicaltrials.gov](http://clinicaltrials.gov)).

### Carfilzomib

Carfilzomib (previously called PR-171; ONYX Pharmaceuticals, Inc., South San Francisco, CA, USA) is a tetrapeptide epoxy-ketone that displays a high degree of selectivity for the  $\beta$ 5 subunit (Demo *et al.* 2007, Kuhn *et al.* 2007, O'Connor *et al.* 2009). The nonspecific activity of carfilzomib against other  $\beta$  subunits and cellular serine proteases appears to be minimal (Arastu-Kapur *et al.* 2011), which may account for the substantially reduced rates of peripheral neuropathy that have been observed in carfilzomib-treated patients (Siegel *et al.* 2013). The epoxy-ketone moiety of carfilzomib forms an irreversible linkage with the  $\beta$ 5 subunit allowing prolonged inhibition of the CT-L activity of the proteasome. However, whether irreversible, vs reversible, proteasome inhibition provides a clear clinical benefit remains unresolved. Carfilzomib is not orally bioavailable and requires intravenous delivery. Recently, carfilzomib was approved by the US FDA for treatment of multiple myeloma patients who have previously been treated with bortezomib (Herndon *et al.* 2013). Clinical testing of carfilzomib has been advanced considerably and it is currently being evaluated in a range of clinical trials in both hematologic and solid tumor malignancies ([clinicaltrials.gov](http://clinicaltrials.gov)). Solid tumors currently being evaluated in clinical trials of carfilzomib include small-cell lung cancer, non-small cell lung cancer (NSCLC), refractory renal cell cancer, and metastatic prostate cancer.

### Oprozomib

Oprozomib (previously called PR-047 and ONX-0912; ONYX Pharmaceuticals, Inc.) is an orally bioavailable analog of carfilzomib (Zhou *et al.* 2009, Chauhan *et al.* 2010). Like carfilzomib, oprozomib is an irreversible epoxy-ketone inhibitor with a high degree of specificity for the  $\beta 5$  subunit. Early stage clinical testing of oprozomib in hematologic malignancies, primarily multiple myeloma, is ongoing. In addition, a Phase I trial of oprozomib in advanced stage solid tumors is also underway ([clinicaltrials.gov](http://clinicaltrials.gov)).

### Marizomib

Marizomib (Nereus Pharmaceuticals, San Diego, CA, USA), also called NPI-0052 or salinosporamide A, is a naturally occurring  $\beta$ -lactone compound that irreversibly inhibits the proteasome (Feling *et al.* 2003, Chauhan *et al.* 2005, Macherla *et al.* 2005). In contrast to the inhibitors described earlier, which primarily inhibit the  $\beta 5$  subunit, marizomib inhibits  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$ . It is interesting that acquired resistance to bortezomib and carfilzomib in *in vitro* models frequently corresponds with overexpression or mutation of the  $\beta 5$  subunit (Kale & Moore 2012, Verbrugge *et al.* 2012), although this has not been observed in patients. Nonetheless, it has been proposed that the ability of marizomib to inhibit multiple proteasome activities ( $\beta 1$ ,  $\beta 2$ , and  $\beta 5$ ) may limit the development of acquired resistance in marizomib-treated patients. Marizomib is currently undergoing Phase I clinical testing in relapsed/refractory multiple myeloma, refractory lymphoma, and advanced solid tumor malignancies. In addition, marizomib in combination with vorinostat is being evaluated in a trial incorporating NSCLC, pancreatic cancer, melanoma, and lymphoma ([clinicaltrials.gov](http://clinicaltrials.gov)).

### Proteasome inhibitors as monotherapy in the treatment of solid tumors

Bortezomib is by far the most extensively evaluated proteasome inhibitor in clinical trials. A wealth of preclinical studies have shown that bortezomib exhibits anti-tumor activity against both hematologic and solid tumor malignancies (Chen & Dou 2010). Among solid tumors, preclinical activity has been observed in the models of NSCLC (Liu *et al.* 2007, Voortman *et al.* 2007a), head and neck squamous cell carcinoma (HNSCC) (Sunwoo *et al.* 2001, Fribley *et al.* 2004, 2006, Li *et al.* 2008), hepatocellular carcinoma (Chen *et al.* 2009),

melanoma (Qin *et al.* 2005), prostate cancer (Williams *et al.* 2003, Nikrad *et al.* 2005), colon cancer (Zhu *et al.* 2005a,b), renal cell carcinoma (Bonner *et al.* 2010), and pancreatic cancer (Nawrocki *et al.* 2002). By contrast, clinical trials of bortezomib treatment (particularly as monotherapy) in solid tumors have generally produced less promising results. As discussed later, several emerging strategies offer hope for improving the efficacy of proteasome inhibitors in the clinic.

Clinical testing of bortezomib in solid tumors has been most extensive in aerodigestive tract tumors, particularly NSCLC and HNSCC. Notably, Phase II studies of bortezomib monotherapy by Li *et al.* (2010) and Besse *et al.* (2012) have failed to demonstrate clinical activity in patients with advanced stage NSCLC. It is possible, however, that specific subtypes of NSCLC may be more responsive to proteasome inhibitors. In a Phase II study, Ramalingam *et al.* (2011) observed modest clinical activity of bortezomib in patients with bronchioloalveolar carcinoma, a NSCLC subtype. In HNSCC, early phase clinical trials by Allen *et al.* (2008) and Gilbert *et al.* (2013) demonstrated pharmacodynamic effects (e.g. NF- $\kappa$ B inhibition) of bortezomib monotherapy, but improvement in clinical outcomes was not observed.

More limited clinical testing of bortezomib monotherapy has been performed in a variety of other solid tumor malignancies. These studies have demonstrated a lack of clinical benefit for monotherapeutic bortezomib in recurrent ovarian cancer (Aghajanian *et al.* 2009), unresectable hepatocellular carcinoma (Kim *et al.* 2012), and metastatic colorectal (Mackay *et al.* 2005), gastric (Shah *et al.* 2011), melanoma (Markovic *et al.* 2005), and prostate (Morris *et al.* 2007) cancers. Interestingly, in preclinical studies of triple-negative breast cancer, cell lines representing the basal-like subtype were found to be more sensitive to proteasome inhibitors than cell lines representing luminal and mesenchymal subtypes (Petrocca *et al.* 2013). This suggests that rigorous stratification of patients according to specific cancer subtypes may be necessary to reveal the therapeutic benefits of proteasome inhibitors in solid tumors.

Although the results of solid tumor clinical trials incorporating bortezomib monotherapy have been somewhat disappointing, several approaches are currently being pursued that may have a major positive impact on further application of proteasome inhibitors. First, as described earlier, a wave of second-generation proteasome inhibitors is currently being developed. As a first-generation inhibitor, the therapeutic efficacy of bortezomib is hindered by several factors, including

nonspecificity and associated adverse toxicities, inherent and acquired resistance, and short-term reversible inhibition. Each of these factors is being addressed in second-generation proteasome inhibitors. For example, it is notable that reduced toxicities are seen with carfilzomib, oprozomib, and delanzomib, which may allow more frequent dosing and more potency *in vivo*. Moreover, agents like carfilzomib and oprozomib are effective in bortezomib-resistance multiple myeloma cell lines and primary multiple myeloma cells from patients refractory to bortezomib treatment (Kuhn *et al.* 2007, Chauhan *et al.* 2010). Second, mechanistic studies have determined that proteasome inhibitors also upregulate the expression of certain proteins (e.g. MCL1) that act to attenuate the killing activity of the agent. Co-targeting with inhibitors of these upregulated anti-apoptotic proteins, or other constitutively expressed pro-survival proteins, is likely to markedly increase the therapeutic efficacies of proteasome inhibitors. Third, as discussed later, molecular targeting of other protein components of the ubiquitin–proteasome system, either alone or in combination with inhibitors of the catalytic core, may be able to circumvent some of the inherent resistance mechanisms of solid tumors.

### Combinatorial strategies incorporating proteasome inhibitors

#### Proteasome inhibitors in combination with conventional chemotherapy or radiation

Results from early-stage clinical testing indicate that bortezomib (and possibly next generation proteasome inhibitors) is unlikely to be effective as a monotherapy against solid tumors in humans. However, co-targeting strategies incorporating proteasome inhibitors with other selective or nonselective agents offer a promising opportunity for improving anti-tumor efficacies (Fig. 3). Numerous preclinical studies have demonstrated synergism between proteasome inhibitors and conventional chemotherapy drugs in both hematologic and solid tumor malignancies (Yang *et al.* 2009). Although combination of bortezomib with conventional chemotherapy in solid tumor clinical trials has yet to realize highly beneficial results, it should be noted that clinical trials in multiple myeloma have shown improvements in efficacy when bortezomib is combined with thalidomide, melphalan, dexamethasone, cyclophosphamide, arsenic trioxide, or doxorubicin (Berenson *et al.* 2007, Orlowski *et al.* 2007, Reece *et al.* 2008, Terpos *et al.* 2008, Popat *et al.* 2009, Chen *et al.* 2011). The evaluation of further drug combinations

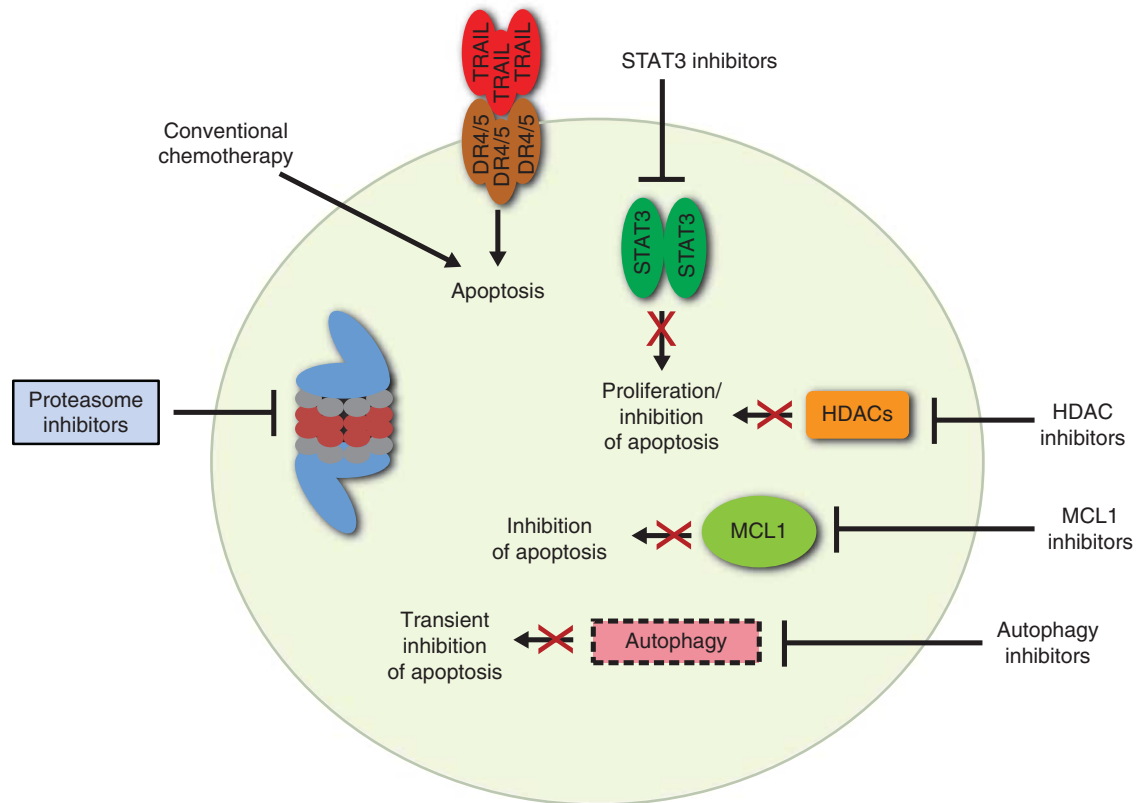
and administration schedules may be necessary to optimize potential synergy in solid tumors.

In NSCLC, Davies *et al.* (2009) have reported a notable survival benefit for bortezomib plus gemcitabine/carboplatin in a Phase II trial of advanced disease, while a Phase II trial by Lara *et al.* (2011) demonstrated enhanced survival by sequential administration of docetaxel and bortezomib. In addition, clinical activity of bortezomib in combination with carboplatin/bevacizumab as first-line therapy was observed by Piperdi *et al.* (2012) in a Phase I/II trial of advanced NSCLC. However, Hoang *et al.* (2013) found only minimal anti-NSCLC activity when bortezomib was combined with vorinostat as a third-line therapy in a Phase II setting.

Bortezomib evaluation in HNSCC has incorporated patients with advanced stage, recurrent, and metastatic disease, and has focused on combination with radiation, conventional chemotherapy, or cetuximab. Pharmacodynamic modulation of NF- $\kappa$ B activity has been detected in patients receiving bortezomib in combination with radiation (Van Waes *et al.* 2005, Pugh *et al.* 2010). Moreover, Kubicek *et al.* (2012) have shown that the combination of bortezomib plus concurrent chemoradiotherapy (cisplatin) is well tolerated in previously treated and radiation-naïve patients. Phase II studies have shown that the addition of bortezomib to irinotecan or docetaxel provides only minimal therapeutic benefit (Chung *et al.* 2010, Gilbert *et al.* 2013). The addition of bortezomib to cetuximab-containing regimens has been evaluated in two Phase I trials. Dudek *et al.* (2009) combined bortezomib with cetuximab in a cohort of patients with solid tumors expressing epidermal growth factor receptors and found that the combination was modestly effective in promoting stable disease in both HNSCC and NSCLC. By contrast, Argiris *et al.* (2011) terminated a trial of bortezomib plus cetuximab and radiation when five of six patients underwent progression earlier than expected. Rather unexpectedly, these early-progressing patients were suspected to be HPV positive. Thus, the hoped-for utility of proteasome inhibitors in the treatment of HPV-positive cancers remains uncertain.

In Phase II studies of metastatic breast cancer, bortezomib in combination with pegylated liposomal doxorubicin or anti-hormone therapy failed to yield any objective clinical responses (Irvin *et al.* 2010). The additions of bortezomib to pegylated liposomal doxorubicin in platinum-resistant ovarian cancer and bortezomib to irinotecan in relapsed/refractory colorectal cancer have also failed to demonstrate any clinical benefit (Kozuch *et al.* 2008, Parma *et al.* 2012). Similarly, in a



**Figure 3**

Co-targeting strategies for anti-cancer therapy. The figure depicts proteins or processes that can be targeted in combination with proteasome inhibitors to improve the efficacy of proteasome inhibitor therapy.

Phase II trial of metastatic prostate cancer, bortezomib in combination with prednisone did not exhibit significant anti-tumor effects (Morris *et al.* 2007). However, Kraft *et al.* (2011) have shown that bortezomib in combination with hormone deprivation therapy exerts a pharmacodynamic effect by changing the slope of prostate-specific antigen upregulation.

### Co-targeting the proteasome and cell surface death receptors

Another opportunity for enhancing the anti-tumor efficacy of proteasome inhibitors may come from co-targeting DR4 and DR5, the cell surface receptors for the death ligand TRAIL (Fig. 3). Treatment of solid tumor cells with proteasome inhibitors has been shown to upregulate DR4 and DR5, enhancing cellular sensitivity to TRAIL (Nikrad *et al.* 2005, Liu *et al.* 2007, Voortman *et al.* 2007b, Shanker *et al.* 2008, Seki *et al.* 2010, Yoshida *et al.* 2011). Targeting of DR4 and DR5 can be achieved through the use of recombinant TRAIL or agonistic antibodies to the receptors.

### Co-targeting the proteasome and MCL1

In addition to inducing the expression of pro-apoptotic proteins that act to mediate cell killing, proteasome inhibitors also induce the expression or activation of proteins that promote cellular survival or proliferation (Fig. 3). Anti-apoptotic MCL1 is markedly upregulated in solid tumor cell lines treated with bortezomib, carfilzomib, or oprozomib (Opferman 2006, Li *et al.* 2008, Zang *et al.* 2012a). Suppression of MCL1 expression using siRNAs/shRNAs or inhibition of MCL1 function using obatoclax can result in synergistic induction of cell death (Perez-Galan *et al.* 2007, 2008, Li *et al.* 2008, Zang *et al.* 2012a). Thus, the combination of bortezomib with a highly selective MCL1 inhibitor represents a promising therapeutic approach.

### Co-targeting the proteasome and STAT3

Bortezomib treatment has also been shown to induce the activation of STAT3 in HNSCC cells, and the inhibition of

STAT3 using a STAT3 decoy oligonucleotide or the naturally occurring compound guggulsterone results in enhanced cell killing following proteasome inhibition (Li *et al.* 2009). Clinical evaluation of proteasome inhibitors in combination with a STAT3 inhibitor awaits the development of a suitable clinical agent to inhibit STAT3.

### Co-targeting the proteasome and autophagy

Proteasome inhibition also promotes the induction of autophagy in a variety of solid tumor cell lines (Ding *et al.* 2007, 2009, Zhu *et al.* 2009, Belloni *et al.* 2010, Li & Johnson 2012). In most cases, the induced autophagy has been shown to temporarily enhance the survival of cells treated with proteasome inhibitors. Inhibition of autophagy using chloroquine can increase the sensitivity of solid tumor cells to bortezomib, carfilzomib, and oprozomib (Fig. 3; Hui *et al.* 2012, Zang *et al.* 2012b). Co-targeting of pro-survival autophagy and the proteasome in clinical trials of solid tumors seems warranted.

### Co-targeting the proteasome and epigenetic-modifying enzymes

Finally, co-targeting with proteasome inhibitors and histone deacetylase (HDAC) inhibitors is currently being intensively investigated (Fig. 3). Yu *et al.* (2003) and Pei *et al.* (2004) were among the first to report potent synergy between proteasome and HDAC inhibitors in preclinical models of leukemia and multiple myeloma, respectively. Subsequently, preclinical synergy of these agents has been reported in a broad range of solid tumor cells, including cell line models representing ovarian (Bazzaro *et al.* 2008, Fang *et al.* 2011, Gatti *et al.* 2012), glioblastoma (Asklund *et al.* 2012), colon (Pitts *et al.* 2009), pancreatic (Bai *et al.* 2006, Spratlin *et al.* 2011), hepatocellular (Spratlin *et al.* 2011), HNSCC (Kim *et al.* 2010), and uterine (Lin *et al.* 2009) cancers. These studies have not been limited to the proteasome inhibitor bortezomib, as synergy of HDAC inhibitors with carfilzomib and marizomib has also been reported (Miller *et al.* 2007, Dasmahapatra *et al.* 2010, 2011). Clinical evaluation of combined treatment with proteasome inhibitors and HDAC inhibitors has been pursued most extensively in multiple myeloma (Hideshima *et al.* 2011). The VANTAGE 088 Phase III trial of multiple myeloma patients reported modestly prolonged progression-free survival (PFS) with vorinostat plus bortezomib (median PFS=7.63 months) vs placebo plus bortezomib (median PFS=6.83 months)

(Dimopoulos *et al.* 2013). The PANORAMA 2 Phase II trial of panobinostat plus bortezomib demonstrated restoration of clinical responses in bortezomib-refractory multiple myeloma (Richardson *et al.* 2013). The potential also exists for combination of proteasome inhibitors with other modulators of epigenetic mechanisms, such as demethylating agents. It is intriguing that bortezomib treatment has been shown to promote hypomethylation of genomic DNA (Liu *et al.* 2008). Moreover, the demethylating agent azacytidine reduces the expression of the multi-drug-resistance transporter MDR/P-gp, a major mediator of bortezomib resistance (Linenberger *et al.* 2001). Evaluation of proteasome inhibitors in combination with azacytidine or decitabine is warranted.

### Other promising targets in the ubiquitin-proteasome system

As depicted in Figs 1 and 2, the degradation of proteins via the ubiquitin-proteasome system is a multi-step and complex process. Thus, opportunities for controlling the degradation of proteins in cancer therapies are not limited to targeting of the 20S catalytic core particle. Numerous other protein and pathway nodes exist and are currently being investigated as sites for potential therapeutic intervention. Below, three brief examples of such efforts are described.

#### Preventing p53 degradation

In view of the ability of p53 to promote either cell cycle arrest or apoptosis, inhibition of p53 degradation in cancer cells is an attractive strategy. Ubiquitination of p53 is mediated by the E3 ligase HDM2. Over the past decade, considerable effort has been invested to identify small molecule inhibitors of HDM2 action or the interaction between HDM2 and p53. The nutlin compounds are the best characterized examples of this category of inhibitors (Vassilev *et al.* 2004, Vassilev 2007, Saha *et al.* 2013). Nutlins disrupt p53/HDM2 interactions, thereby inhibiting p53 ubiquitination. Nutlin treatment leads to p53 accumulation and induction of cell arrest and apoptosis. Importantly, nutlin induction of cell cycle arrest and/or apoptosis is restricted to cells that express wild-type p53, supporting the mechanism of action of these agents, but also highlighting a limitation of nutlins (and similar inhibitors) for broad use as anti-cancer drugs. More recently, a large number of novel HDM2 antagonists have been identified and are undergoing preclinical evaluation (Shen *et al.* 2013).

## Inhibition of neddylation

Restoration of the CDK inhibitor p27 in cancer cells has the potential benefit of inhibiting entry into the cell cycle, thereby slowing the growth of tumor cells. The p27 protein is subjected to ubiquitination and proteasomal degradation through the action of the SCF<sup>Skp2</sup> E3 ligase protein complex. Interestingly, activation of this complex requires conjugation with the ubiquitin-like polypeptide NEDD8 via a process termed neddylation (Merlet *et al.* 2009). Thus, inhibition of neddylation can be used to prevent SCF<sup>Skp2</sup> activation and p27 loss. Similar to the activation of ubiquitin by the E1 enzyme Ube1, nedd8 is activated by the nedd8-activating enzyme (NAE; Soucy *et al.* 2010). Soucy *et al.* (2009) have identified a small molecule inhibitor of NAE called MLN4924. Treatment with MLN4924 promotes S-phase defects, apoptosis, and growth inhibition of solid tumor xenografts in mice (Soucy *et al.* 2009, Brownell *et al.* 2010, Milhollen *et al.* 2011). MLN4924 is currently undergoing early phase clinical testing.

## Inhibition of Dubs

The degradation of proteins by the 20S catalytic core is preceded by protein deubiquitination. Deubiquitination is accomplished by the Dub family of enzymes (Fig. 3). Roughly, 100 Dubs have been identified, with varying substrate specificities (Fraile *et al.* 2012). Thus, inhibition of selective Dubs may prove useful for altering the balance of pro- vs anti-apoptotic proteins in the cell. Chauhan *et al.* (2012) have identified the small molecule P5091 as an inhibitor of the Dub enzyme USP7. P501 induces apoptosis in bortezomib-refractory multiple myeloma cells and inhibits the growth of myeloma tumors *in vivo* (Chauhan *et al.* 2012). Recently, Tian *et al.* (2014) have shown that the compound b-AP15 acts to inhibit the deubiquitinating activity of USP14 and UCHL5, but does not impact overall proteasome activity. b-AP15 induces cell death in multiple myeloma cell lines and primary specimens and is able to overcome bortezomib resistance. Moreover, b-AP15 was shown to synergize with lenalidomide, dexamethasone, and vorinostat, and inhibit the growth of multiple myeloma xenograft tumors. Although early in preclinical development, additional inhibitors of Dub enzymes have also recently been identified (Shen *et al.* 2013).

## Future considerations

The promise of proteasome inhibitors in the treatment of solid tumor malignancies has yet to be realized. At present,

most clinical studies in solid tumors have been carried out using bortezomib the first-generation proteasome inhibitor. The second-generation compounds currently under development and evaluation offer the potential for improvements in potencies, selectivities, and other drug-like properties. Nonetheless, it seems likely that monotherapeutic application of proteasome inhibitors may have only limited success in solid tumors. It will be important to continue the development of co-treatment strategies that simultaneously target the proteasome and other proteins/pathways that suppress apoptosis or cell cycle arrest. Fortunately, there are numerous opportunities for co-targeting strategies, and multiple examples of synergistic anti-cancer drug combinations have been reported in preclinical solid tumor models. Whether such synergism will be demonstrated in human clinical trials warrants investigation. In addition, future studies should continue to be aimed at developing small molecule regulators of components of the ubiquitin–proteasome system that are distinct from the proteasome complex. The opportunities in this regard are tremendous, and are currently highlighted by efforts to develop inhibitors of the E1, E2, E3, and Dub enzymes. It is likely that targeting of these different enzymes may achieve either pan-inhibition or highly selective inhibition of protein degradation, with the therapeutic value of these endpoints yet to be determined.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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