Antiproteasomal agents in rectal cancer

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Colorectal cancer (CRC) is a prevalent and highly morbid condition. An improved understanding of the molecular pathogenesis of CRC in recent years has led to novel therapies complementing traditional chemotherapy, radiotherapy, and surgery. As in other cancers, it has become clear that the ubiquitin–proteasome system represents important cellular machinery that plays a complex role in the carcinogenesis of CRC, and may be a promising target for modulation in the treatment of CRC. In particular, there has been promise in targeting nuclear factor-κB and cell-cycle pathways in CRC through proteasome inhibition. Proteasome inhibition may be an important means of sensitizing cancers to traditional chemotherapy and radiotherapy through these pathways. In this review, we outline the basic science of the ubiquitin–proteasome system in CRC pathogenesis, highlight the use of proteasome inhibitors in cancers other than CRCs, and weigh the accumulating evidence and data, both preclinical and clinical, for the use of proteasome inhibition in CRC. Furthermore, we review the emerging evidence of proteasome inhibition as a possible radiosensitizing agent in rectal cancer and elucidate some possible future directions for this novel therapeutic option.
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Introduction

The incidence of colorectal cancer (CRC) worldwide is approximately 1 000 000 per year [1]. The 5-year survival rate in the United States is only 61% [2]. Much is now understood about the carcinogenesis and pathophysiology of CRC and there is a growing pool of novel therapeutic tools available for treatment. These novel therapeutic tools and strategies, derived from a better understanding of the biology of CRC, have resulted in new combination therapies in CRC and other malignancies. One of these fairly recently discovered strategies targets the proteasome, which plays a central role in the pathophysiology of CRC.

The proteasome is a multiprotein cellular complex that is involved in the modulation or degradation of cellular proteins [3]. It was discovered by Ciechanover, Hershko, and Rose, who were awarded the Nobel Prize in Chemistry in 2004 for this discovery. The proteasome is involved in virtually every cellular function. Its crucial role in the regulation of proteins relevant to carcinogenesis, such as apoptosis, proliferation, and metastasis [4], has led to the development of novel anticancer strategies targeting this system. Additional important cancer-relevant functions regulated by the proteasome include cell cycle [5], immunological function [6], angiogenesis [7], and cell adhesion [8]. Owing to the involvement in the processes relevant for cell survival, proteasome function is a requirement for the maintenance of not only normal cells, but also neoplastic cells. To be recognized by the proteasome for processing, a protein substrate needs to be linked to the protein ubiquitin, a chain of 76 amino acids [3]. Ubiquitin linkage is completed in subsequent reactions carried out by different enzymes. They are named ubiquitin-activating enzymes or E1, ubiquitin-conjugating enzymes or E2, and ubiquitin ligases or E3, respectively. The proteasome has three enzymatic activities defined as chymotryptic, tryptic, and glutamyl–peptidyl activity. The importance of each activity varies depending on the substrate. Protein ubiquitination is a reversible process and there exist deubiquitinating enzymes that catalyze the deubiquitination of the proteins preventing their degradation.

In addition to protein degradation, the multitude of carcinogenesis-relevant biological functions make the ubiquitin–proteasome complex an important anticancer target, using antiproteasomal agents. This review will focus on the importance of proteasomes on CRC pathogenesis, put proteasomal inhibitor strategies used to treat CRC in perspective *vis-à-vis* their use in other cancers, and present the available preclinical and clinical data on the treatment of CRC.

The proteasome and its importance in colorectal cancer pathogenesis

The proteasome is an assemblage of proteins whose chief function is to degrade other proteins in cells that are marked in a particular way so as to signal their readiness for degradation, usually through the addition of ubiquitin

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(a small polypeptide) groups to certain moieties in the proteins to be destroyed [3]. The proteasome degrades many important proteins involved in the regulation of the cell cycle, including cyclins A, B, D, and E [9–11] and p21 [12], p27 [13], and p53 [14]. The ubiquitination and degradation of p53 by the proteasome allow for the disinhibition of cyclin-dependent kinases, such as the transcription factor E2F, which contributes to the expression of genes that lead to DNA synthesis [15]. p73, which is related to p53, and ASPP-53BP2, a p53 cofactor, are also proteasome substrates [16]. Other gene products that have protean effects on cell cycle and are more proximal in cell-cycle pathways include c-myc [17], which is also commonly mutated in solid tumors [18,19]. This product is also degraded by the proteasome.

The nuclear factor-κB (NF-κB) pathway is also regulated by the ubiquitin–proteasome system (UPS). NF-κB is a transcription factor that, when active, promotes transcription of genes with a κB response element, such as cytokine interleukin-6, which promotes DNA synthesis and proliferation [20,21], angiogenic factor vascular endothelial growth factor, cell adhesion molecules (e.g. intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), and cell survival factors (e.g. Bcl-2) [22–24]. IκB, the inhibitor of NF-κB, is a substrate of the proteasome [25]. Proteasomal degradation of IκB leads to the activation of NF-κB. Multiple myeloma cells have been shown to have constitutively active NF-κB [26], which is protective against apoptosis. In addition, increased cell adhesion molecule expression in these cells may contribute to cell proliferation and chemoresistance [20,27]. NF-κB has also been found to be constitutively activated in solid organ malignancies, for example, in pancreatic adenocarcinoma [28]. In CRC, β-TrCP, which is the ubiquitin ligase that ubiquitinates IκB, is upregulated because of β-catenin activation [29]. NF-κB upregulation in colorectal carcinogenesis has been described [30–33], and increased activity may partly be because of an increase in β-catenin activation and stabilization with β-TrCP as a downstream target of β-catenin. Antiapoptotic activity of NF-κB induced by treatment with chemotherapy or radiation therapy has been shown to promote survival in CRC models by inhibiting apoptosis [30,34,35].

Although the UPS generally inhibits neoplasia with regard to its role in the cell cycle and the NF-κB pathway, the UPS has divergent functions in other important cellular processes implicated in carcinogenesis. For example, apoptosis is also heavily reliant on the UPS for regulation, but the UPS has opposing roles at various points in the apoptotic pathway. As described above, the UPS has some indirect antiapoptotic effects through its mediation of NF-κB activity. Proteasomal degradation by c-Fos is also an important early step in apoptosis [36]. Proteins in the Bcl-2 family, including Bcl-2, Bax, and Bcl-xL, all key players in the apoptosis response, are regulated by the proteasome in a differential manner [37,38]. Akt, an important kinase that inhibits apoptosis is a proteasome substrate as well [39,40]. In addition to targeting Bad (a member of the Bcl-2 family) and caspase 9 and MDM2, which is an E3 ligase for p53, Akt has many other diverse targets that promote cell proliferation, including mammalian target of rapamycin (mTOR), IκB kinase and glycogen synthase kinase 3β, and transcription factor forkhead in rhabdomyosarcoma [41]. Although the role of the UPS is complex, it is clear that antiapoptotic genes that are modified by the UPS are often unregulated in cancer [42]. In addition, Bcl-2 overexpression can contribute to chemoresistance [43].

The Wnt/β-catenin pathway is a key regulator of the normal development of colorectal epithelium [44,45] and its interaction with the UPS is similarly divalent. When Wnt is associated with Frizzled, the proteasomal degradation of β-catenin is inhibited. This allows β-catenin, along with the transcription factor TCF4, to promote transcription of gene products involved in cell-cycle progression and proliferation, such as cyclin D, c-myc, Akt, c-jun, and fra-1 [46–49]. β-catenin activity is higher in the dividing cells in colonic crypts compared with villus tips in which cells are more differentiated [50]. The stabilization of β-catenin and prevention of its proteasomal degradation are, in fact, a key step in both familial and sporadic CRC carcinogenesis [51]. Adenomatous polyposis coli (APC) is the gene that normally phosphorylates β-catenin and leads to its ubiquitination and subsequent destruction. APC is mutated in familial adenomatous polyposis, a genetic colon cancer syndrome, and is also found to be mutated in 85–90% of sporadic colon cancers. Mutation of APC has been shown to be one of the first steps leading to an aberrant crypt focus, a precursor lesion of CRC [52,53]. In contrast, Hath1 is a gene product regulated by Wnt, which promotes cell differentiation in colorectal mucosa [54–56], and it can be ubiquitinated and then degraded by the proteasome [57]. Hath1 is downregulated in colonic neoplastic tissues compared with normal adult human colonic tissue [56]. Therefore, the UPS may inhibit carcinogenesis *vis-à-vis* its role in degrading β-catenin, but it may promote carcinogenesis *vis-à-vis* its role in degrading Hath1.

Other pathways regulated by proteasomal substrates are pathogenic in CRC. These include the TGF-β pathway, which includes Smad4 (also known as deleted in pancreatic cancer 4) as a proteasomal substrate [58] and hypoxia-inducible factor 1 (HIF-1)/von Hippel–Lindau (VHL). When cells are in an environment of normoxia, VHL ubiquitinates HIF-1 leading to its degradation. When HIF-1 is not degraded, it leads to the transcription of vascular endothelial growth factor, erythropoietin, and anerobic metabolism genes. VHL mutation is responsible for the familial von Hippel–Lindau syndrome in which renal carcinomas commonly arise. Interestingly, VHL mutations occur in about 10% of CRCs [59]. Finally, changes in proteasomal components themselves can contribute to

CRC pathogenesis, including the proteasome subunit PSMA7, the overexpression of which is associated with tumorigenicity and migration [60].

The therapeutic implications of proteasome inhibition in cancer

The great number and importance of pathways described above that contribute to oncogenesis and that are strongly regulated by the proteasome provide a rationale for modifying the proteasome pharmacologically as a method to prevent growth and spread of cancer. Earlier, we have highlighted that a-priori prediction of effects of proteasome inhibition is difficult because the UPS has competing roles in some key cellular processes involved in neoplasia. Despite this, many preclinical studies and now clinical studies have provided data to support the use of proteasome inhibitors for a variety of solid and liquid tumors.

The first proteasome inhibitors were peptide aldehydes that had been used as protease inhibitors and then were found to have nonspecific inhibitory activity against the proteasome [61]. The use of boronic acid instead of aldehyde and the elimination of one amino acid from the structure produced molecules that are potent and specific inhibitors of proteasome function. The dipeptide boronic acid molecule known as bortezomib (also known as PS-341) inhibits the proteasome (Ki of approximately 0.6 nmol/l) but has little inhibitory effect on cellular proteases for chymotrypsin, Ki = 320 nmol/l and for thrombin, Ki = 13 000 nmol/l [62]. Other proteasome inhibitors include NPI-0052, MG-132, and carfilzomib (Table 1).

Many cancers have been shown to be sensitive to the use of proteasome inhibitors as antineoplastic agents, including hematologic malignancies such as multiple myeloma and lymphoma [70], and also solid tumors such as breast, prostate, head and neck cancers, and CRC [71–76]. Initial cell line screens against solid tumors showed that bortezomib was an active agent [75]. Since then, there have been many studies showing in-vitro efficacy of bortezomib and other proteasome inhibitors against neoplastic tissues. Data have shown that the effect can be through many of the carcinogenic pathways described earlier and known to have proteasomal substrates.

Studies on using bortezomib in multiple myeloma have shown efficacy both as a monotherapy and in combination with other drugs at the preclinical and clinical levels. Inhibition of NF-κB seems to be an important mechanism for this efficacy [77,78]. Furthermore, there is a strong effect through the inhibition of cell-cycle progression [79]. Efficacy in some other cancers may be due more to the inhibition of degradation of proapoptotic factors. Bold *et al.* [43] showed that MIA-PaCa-2 pancreatic cancer cells may have a gemcitabine resistance related to Bcl-2 expression, which can be abrogated by bortezomib. In another study, bortezomib and paclitaxel were shown to promote Bcl-xl upregulation in pancreatic cancer [42]. Synergy with camptothecins, topoisomerase inhibitors, and platinum-based therapies has also been shown [34,80,81]. In gastric carcinoma cell lines, the proteasome inhibitors PSI and PS-341 were shown to be synergistic with histone deacetylase inhibitors (m-carboxycinnamic acid bis-hydroxamide and FK228) in terms of inducing apoptosis [82]. This was also shown in colonic carcinoma cell lines in the same study. The proteasome inhibitor MG-132 can sensitize cervical cancer cell lines to the apoptotic stimulus rhTRAIL (recombinant human tumor necrosis factor-related apoptosis inducing ligand); the apoptosis that is induced seems to be independent of the elevated levels of p53 that ensue after proteasome inhibition [83].

Proteasome inhibition promotes radiation sensitivity

The observation that the NF-κB pathway is important for proteasome inhibitor efficacy provides a rationale for the use of proteasome inhibitors synergistically with traditional cancer therapies, as described above. Indeed, not only have proteasome inhibitors been shown to be useful chemosensitizing agents, but they have also been shown to be very useful radiosensitizing agents. For example, Hodgkin's lymphoma cells can be sensitized to ionizing radiation by MG-132 [84]; so too are the prostate cancer cell lines sensitized with MG-132 [85] or bortezomib [86]. However, Hodgkin's lymphoma cells were radiosensitized despite no change in the levels of constitutively active NF-κB. In murine and cell culture models of nonsmall cell lung cancer, MG-132 was also radiosensitizing (and led to delayed tumor regrowth) and the NF-κB activity was evidently lower in proteasome inhibitors-treated cancers [87]. The researchers also showed that induction of manganese superoxide dismutase, a cytoprotective antioxidant enzyme, is inhibited by MG-132 after radiation treatment; this is an NF-κB-dependent

Table 1 Proteasome inhibitors

bortezomib [a.k.a. PS-341, Velcade (R)]
NPI-0052 (a.k.a. Salinosporamide A)
MG-132
carfilzomib (a.k.a. PR-171)
MLN9708
PR-047
gliotoxin
CEP-18770
dihydroeponemycin
lactacystin
BzLLLCOCHO
disulfiram
curcumin
PSI

Proteasomes are enzymes with a complex structure and function that regulate proteins involved in cellular processes, including cell-cycle progression, transcription, and apoptosis. Inhibition of proteasomes has been shown to be an efficacious adjunct to conventional anticancer therapy. In 2003, bortezomib was the first proteasome inhibitor to be approved for use in the United States. Since then many agents have been introduced to clinical trials [63–69].
a.k.a., also known as.

mechanism of cell repair in ionizing radiation, which could help explain radiosensitization. Bortezomib can also radiosensitize cervical cancer cell lines, but this effect seems to be found only in NF-κB-dependent cell lines, for example SiHa cells but not in HeLa cells [88]. Bortezomib reduces Akt signaling and radiosensitizes SQ20B head and neck cancer cells [89]; it is notable that in another cancer model (pancreatic), Akt signaling is actually increased with the use of proteasome inhibitor [90], showing that proteasome inhibitor effects are dependent on a particular genotype or gene transcription profile of the underlying malignant cells.

With encouraging preclinical data, Van Waes *et al.* [91] conducted a clinical trial, which showed that bortezomib could synergistically reduce radiation-induced NF-κB activity, using radiotherapy in combination with bortezomib in previously irradiated recurrent head and neck squamous cell carcinoma. The maximum tolerated dose (MTD) for bortezomib in this heavily pretreated group, because of its side effects including hyponatremia, hypotension, and mucositis, seemed to be approximately 0.6 mg/m^2/dose although they noticed a more potent effect on NF-κB at 0.9 mg/m^2. In contrast to these studies, some cancers do not exhibit increased radiosensitivity with the use of proteasome inhibitor. For example, even NPI-0052, which is a very potent proteasome inhibitor, did not sensitize glioma xenografts to fractionated radiation; along with temozolamide, NPI-0052 radiosensitized only one cell line, which had a mutated p53 [92]. Despite mixed data on gliomas, a phase I trial enrolling patients with central nervous system malignancies (many of whom had high grade gliomas) showed that bortezomib in combination with radiotherapy and temozolamide was well tolerated at the typical systemic dose (i.e. that usually used in myeloma) of 1.3 mg/m^2 [93].

Other cancer models have shown promising results. The use of proteasome inhibitor in small cell lung cancer cells which showed increased levels of c-myc led to increased apoptosis [94]. The researchers did not measure p27 in this experiment, but they suggested that upregulation of p27 and c-myc may lead to apoptosis. Studies in multiple myeloma, MDA-MB-231 breast cancer cells, and LoVo colon cancer cells with bortezomib or CEP1612 (a different proteasome inhibitor) have shown increased p27 levels after proteasome inhibition [20,34,95]. In FH109 lung fibroblasts, p53 increases with the use of proteasome inhibitor and these cells are arrested at G1/S [96].

Although proteasome inhibitors do have modest activity as monotherapy in some cancers, the chief strength of proteasome inhibitors is that they are effective chemosensitizing and radiosensitizing agents. Much of the basic research about proteasome function would predict that this sensitization of cancer cells to traditional therapies would be mediated through p53 and the cell cycle or through the NF-κB pathway. Indeed, this seems to be the case in at least some cancers. However, researchers have also convincingly showed that these effects seem to be independent of the above pathways in certain situations.

Preclinical support for the use of proteasome inhibitors in colorectal cancer

As in myeloma, proteasome inhibitors in preclinical studies of CRC have activity as monotherapy. Colon cancer cell growth is inhibited and apoptosis is induced by MG-132 [97], and the growth inhibition is associated with effects on the NF-κB pathway. Coquelle *et al.* [98] showed that bortezomib causes growth inhibition in colorectal cell cultures through effects on the NF-κB pathway. Duplication of these findings *vis-à-vis* effects on CRC cell growth and apoptosis by proteasome inhibition have been documented with other proteasome inhibitors, such as curcumin in human colon cancer HCT-116 and SW480 cell lines [63].

Although the NF-κB pathway has been implicated as having major importance in the mechanism of proteasome inhibitor effects on CRC, multiple studies have shown other pathways involved in the action of proteasome inhibitor on the inhibition of CRC cell growth.

MG-132 arrests cells from the HCT-116 CRC cell line at the G2/M phase, which is associated with the blockade of p53 degradation and accumulation of cyclin B, cyclin A, and p21 [99]. However, HCT-116 (p53−/−) and HCT116 (p21−/−) cells were observed to have similar effects from MG-132, arguing that these targets are nonessential for proteasome inhibitor action.

In CRC cells, proteasome inhibition seems to cause the activation of mitochondrial pathways of apoptosis that rely on effects on p53, PUMA, and Bax [100]. Another study showed that bortezomib causes downregulation of SKP2 and accumulation of p27Kip1, which is a cyclin-dependent kinase inhibitor [101]. In this study, XIAP, cIAP1, and survivin were downregulated in CRC cells (or CRC cell line xenografts) treated with bortezomib also implicating a mitochondrial pathway of apoptosis with the activation of caspases in using proteasome inhibitor in CRC. The autophagy pathway is also involved. Growth inhibition of cells from CRC cell line HT-29 by MG-132 is associated with the activation of autophagy as shown by the formation of LC3(+) autophagic bodies [102].

In addition, the decreased proliferation of HT-29 and SW1116 CRC cell lines in response to MG-132 is associated with reduced phosphorylation of mTOR and phosphorylation of its downstream targets 4E-BP1 and p70/p85 S6 kinases. This was also associated with the inhibition of protein translation, which could be reproduced by direct mTOR inhibition, suggesting that

the effects on protein translation might be mediated by the reduced phosphorylation of mTOR from MG-132 [103].

A recent study using a systems approach to identify mechanistically important targets of proteasome inhibitors in HCT-116 cells confirmed that mTOR and protein translation were affected by proteasome inhibitors, and p53 [104]. This study, conducted by a group at Millennium Pharmaceuticals, the developer of bortezomib, used an siRNA screen to identify genes whose knockdown mitigates the effects of bortezomib, thus showing their importance for bortezomib to have an effect on CRC cells. The researchers found 100 genes including those related to mTOR and p53 and polyamines, DNA damage repair, endoplasmic reticulum stress, and myc stabilization. Although mTOR was identified again as in the study discussed above, the researchers suggested that proteasome inhibitor effects on mTOR paradoxically cause an increased protein translation in cancer cells, but that this phenotype depends on the levels and activity of mTOR targets such as 4E-BP1. Some of the genes identified in this study had already been known to be regulated by the proteasome and involved in the anticancer mechanisms of proteasome inhibitors; however, the unbiased approach of using a screen provided the advantage of identifying novel pathways as well. Interestingly, they did not identify the involvement of ROS, NF-κB, activator protein-1, or some cell-cycle proteins.

Other genes found to be affected by proteasome inhibition include the inducible isoform of heat shock protein 70, lactate dehydrogenase B, aldo-keto reductase family 1 member B10 in Caco-2 and HRT-18 CRC cells [105]; Hsp70B', a heat shock protein, in HT-29 and CRL-1809 but not the SW-480 CRC cell lines [106]; members of the bone morphogenetic protein family which in turn affect Smad1/5/8 and p21(Waf1/Cip1), and p27(Kip1) in SW1116 and HT-29 CRC cell lines [107].

It is clear from the multitude of preclinical studies that there are many pathways contributing to the efficacy of proteasome inhibitor use in CRC. In addition to the studies of proteasome inhibitors as monotherapy, much work has been done on the use of proteasome inhibitors in conjunction with chemotherapy or radiotherapy for CRC, as has been shown to be an effective strategy in other cancers. In 2001, Russo *et al.* [35] showed that proteasome inhibition leads to an increased radiosensitivity of CRCs by inhibiting activation of NF-κB in LoVo cells, a CRC cell line. Doses of radiation ranged between 2 and 10 Gy, a dose that is a similar to that used for short-course radiotherapy in rectal cancer, a protocol of 25 Gy total dose, given in five fractionated doses of 5 Gy each [108]. The use of proteasome inhibitors as agents that sensitize tumors to traditional chemotherapies and radiotherapy therefore has a strong foundation in the preclinical data for CRC as in other cancers. Combination therapy using proteasome inhibitors ideally promotes chemosensitivity and allows a dose reduction of traditional therapies, avoiding dangerous toxicities associated with them. In other words, the use of proteasome inhibitors may decrease the therapeutic threshold of cancer to these traditional therapies. An attractive option in rectal cancer is using proteasome inhibitors to indiscriminately sensitize cells to chemotherapy/radiotherapy, and then using radiotherapy to treat tumors in a localized manner, the efficacy of which would be enhanced by proteasome inhibition. This could also potentially lead to the use of proteasome inhibitors for recurrent cancers that have developed resistance to our best therapies (particularly those in which the acquired resistance is NF-κB dependent) because proteasome inhibitors may resensitize these tumors. For example, proteasome inhibitors have also been shown to resensitize colon cancer cells that have become resistant to TRAIL-mediated apoptosis [109].

Similarly, pretreatment of human CRC cells with PS-341 before giving topoisomerase I inhibitor CPT-11 results in a synergistic level of growth inhibition [34]. In 2006, proteasome inhibitor NPI-0052 was shown to increase the activity of conventional chemotherapy in CRC [31,110]. More recently, vorinostat and bortezomib were shown to be efficacious when used together in CRC cell cultures [110].

In addition to these studies on the use of proteasome inhibitor against colon cancer, it has been suggested that proteasome inhibition plays a key role in the antineoplastic effects of other agents commonly known to be effective in the treatment or prevention of CRC. For example, NSAIDs are known to be antineoplastic and tumor suppressive [111,112]. There have been many suggestions regarding the cause for these properties and it has also been noted that there is a COX-independent mechanism for these effects on colorectal cells. IκB kinase inhibition, peroxisome proliferator-activated receptors activation, β-catenin inhibition, and spermidine/spermine N1-acetyltransferase induction have all been suggested as playing mechanistic roles [113–117]. However, proteasome inhibition has also been found to be involved in aspirin-induced apoptosis [118].

Use of proteasome inhibitors in colorectal cancer in humans and future directions

Although the preclinical data for bortezomib strongly support its use in CRC, particularly for the sensitization of cancer cells to other therapeutics, the clinical data thus far have been mixed. Unfortunately, the quality of the data has been limited by use only in phase I or II trials that are largely not controlled, blinded, or appropriately powered to detect a difference between proteasome inhibitor use and standard therapy. In 2005, Mackay *et al.* [119] published a phase II study using bortezomib (1.3 mg/m^2 on days 1, 4, 8, and 11 of a 21-day cycle) as

a single agent against metastatic colon cancer. This trial enrolled 19 patients, 14 of whom either progressed after one to four cycles and two of whom did not receive therapy past the second cycle because of toxicity; three patients were not evaluable. There were several grade 3 toxicities; the most common ones were lymphopenia, abdominal pain/cramping, vomiting, and sensory neuropathy. In this study, the researchers also carried out immunohistochemistry for a functional assessment of certain substrates of the proteasome. They showed that HIF-1α and CAIX (carbonic anhydrase IX, a transcriptional target of HIF-1α that is involved in anerobic metabolism) levels changed after treatment. They were unable to show a change in the levels of other substrates of the proteasome including p53, NF-κB, or IκB. The researchers used a xenograft model to show that HIF-1α transcriptional targets (e.g. CAIX) were unexpectedly lower in hypoxic regions of tumors posttreatment (compared with normoxic regions), suggesting that although HIF-1α may increase with bortezomib, in hypoxic areas of tumors bortezomib may disrupt the tumor response to hypoxia, which could potentially explain the synergy between proteasome inhibition and ionizing radiation [35].

Other studies have used bortezomib combined with another anticancer agent. After Ryan *et al.* [120] published a phase I trial on the safety of bortezomib and irinotecan for patients with advanced solid tumors, 23 of whom were patients with colorectal cancer, a trial of bortezomib with or without irinotecan in CRC was published in 2008 by Kozuch *et al.* [121]. In this phase II study, 102 patients with relapsed or refractory CRC (inoperable, locally advanced, or metastatic) were treated, 45 of whom were randomized to bortezomib and 57 of whom were randomized to bortezomib and irinotecan. This study was terminated at an interim analysis because of inadequate activity. Two partial responses (3.5%) were seen in the combination arm and no responses were seen in the bortezomib-only arm.

In 2009, the EORTC (European Organisation for Research and Treatment of Cancer) published a phase I study on bortezomib in combination with oxaliplatin, leucovorin, and 5-fluorouracil (5-FU) in patients with advanced CRC who had not been treated earlier for metastatic disease [122]. This represents the addition of a biologic (bortezomib) to the established regimen of FOLFOX-4. This trial enrolled 16 patients. Three of them received less than two cycles. Of the other 13 patients, six had a partial response and one had a complete response. The researchers concluded that this regimen may deserve future study. They found that a dose of 1 mg/m^2 was the MTD because only one dose-limiting toxicity (DLT) was observed among six patients at this dose. Grade 3 toxicities at higher doses included neutropenia, neuropathy, fatigue, weakness, and diarrhea.

A phase I study was recently published using bortezomib in combination with standard 5-FU and external-beam radiation therapy [123]. The population studied included patients with locally advanced rectal adenocarcinoma. Ten patients were enrolled and nine completed the study. Bortezomib was given biweekly for 5.5 weeks on weeks 1, 2, 4, and 5. The dose of bortezomib was not increased above 1.0 mg/m^2 because of grade 3 diarrhea. At these doses, no grade 3 or 4 neuropathy or hematologic toxicity was observed. MTD was determined to be 0.7 mg/m^2 due to diarrhea. Histopathological evaluation of the nine patients who completed the study and underwent resection showed one pathologic complete response, one microscopic residual disease, and seven cases of gross residual disease. In this study, the researchers were able to measure the NF-κB mRNA copy number in the biopsy specimens. Interestingly, the patient with the pathologic complete response had an increase in mRNA copy number of NF-κB after bortezomib treatment. Unfortunately, the MTD found in this study (0.7 mg/m^2) was the same as that determined in another trial of PS-341 with 5-FU in advanced solid tumors, many of which were colorectal primaries [124] with similar DLTs. This is unfortunately substantially lower than the standard dose in multiple myeloma (1.3 mg/m^2). O'Neil *et al.* [123] pointed out that at a dose of 1.3 mg/m^2, proteasome activity is 60% inhibited in primates [71]. They stated that a dose of 0.7 mg/m^2 may not be a 'biologically meaningful' dose and therefore a phase II trial with the above regimen is not being pursued.

It is very difficult to draw conclusions from these studies about the clinical efficacy of bortezomib. Phase I and II trials, by definition, are not designed to rigorously show a benefit over standard therapy. These are open-label trials, largely without a control group, on populations of patients most of whom have had disease progression on standard therapy. It is clear from the above studies that bortezomib alone is not an active agent against advanced CRC. However, bortezomib with other chemotherapeutics or with radiotherapy may be combinations which provide a benefit for patients with CRC. The O'Neil *et al.* [123] trial confirmed an unfortunately low MTD for bortezomib when used with 5-FU; but a different regimen using proteasome inhibition and radiotherapy may be quite viable. The preclinical data have shown that proteasome inhibition has synergistic effects with radiation and chemotherapy in CRC. The promise of suppressing NF-κB activity to enhance apoptosis in tumors undergoing treatment with localized therapy (i.e. radiotherapy) in rectal cancer should drive further studies looking at combination therapies, also for cancers other than CRC. Van Waes *et al.* [91] showed that this approach is plausible and efficacious in head and neck cancers.

In rectal cancer, a very promising approach may be to combine proteasome inhibitor therapy with short-course preoperative radiotherapy, currently used more frequently in Europe than in the United States. Short-course preoperative radiotherapy is hypofractionated and results

in a lower total dosage of irradiation than contemporary conventional neoadjuvant radiotherapy (which is administered in conjunction with 5-FU as chemoradiotherapy). It is 5×5 Gy for a total dose of 25 Gy as opposed to 1.6 or 1.8 Gy twice daily for a total dose of 40–60 Gy. In short-course preoperative radiotherapy, surgery is typically performed within 3 to 10 days of the completion of radiation whereas surgery is performed usually 4–6 weeks after conventional chemoradiation with 5-FU (as was done in the O'Neil *et al.* [123] trial). Preoperative chemoradiation is the current standard of care in the United States for T3 or N+ rectal cancers, and this approach leads to a significant amount of downstaging at surgery [125,126]. A publication from a group in Poland showed that short-course preoperative radiotherapy has equivalent outcomes with regard to local control, survival, and sphincter preservation [126]. An advantage of short-course preoperative radiotherapy is that because surgery (total mesorectal excision, TME) shortly follows radiotherapy, there is accurate pathologic staging [127,128]. Russo *et al*. showed that proteasome inhibition sensitizes colorectal cancer cells (evidenced by apoptosis) to increasing dosages of radiation, including those levels (5 Gy) used in hypofractionated short-course radiation therapy. Short-course pre-operative radiotherapy avoids the toxicity associated with concurrent 5-FU administration. Combining short-course pre-operative radiotherapy with proteasome inhibitor therapy alone may be a strategy that could further improve local control without increasing dose limiting toxicity related to 5-FU.

In fact, a recent phase I trial of bortezomib in combination with radiotherapy for advanced solid tumors showed that the MTD in this context was at least 1.6 mg/m^2, given once weekly with concurrent radiation, 16 fractions for a total of 40 Gy [129]. The only toxicity experienced by patients in this trial that could be considered a DLT was grade 3 urosepsis, which was not thought to be related to bortezomib. Patients did experience lymphopenia, nausea, diarrhea, fatigue, and infection without neutropenia, but none of these met the criteria to be considered a DLT. Trials such as this, the Van Waes *et al.* [91] trial, and the central nervous system malignancy trial [93] show that the use of proteasome inhibitors as radiosensitizers is feasible and deserves further study, and doing so in the context of short-course preoperative radiotherapy could be a viable start.

In short, further studies on the mechanisms of proteasome inhibition and their role in combination therapy are warranted at both the preclinical and clinical levels. The mechanisms underlying the pleiotropic effects of proteasome inhibitors and which effects are most important in different cancers are still incompletely elucidated. Further study will help to show the genetic and epigenetic profiles of those cancers that are particularly sensitive to the use of proteasome inhibitor. Many of the preclinical studies have shown heterogeneity among various CRC cell lines in their responses to proteasome inhibitors and it is clear that some other types of cancers (e.g. glioma, cervical cancer) are variably susceptible to proteasome inhibitor use. With a better understanding of important pathways in the effects of proteasome inhibitors, we will also be able to determine which therapy modalities may be most effective in combination with proteasome inhibitors. Development of other proteasome inhibitors is another important avenue of research. Finally, there are several very promising avenues for further clinical studies, particularly in the use of proteasome inhibitors as radiosensitizers in rectal cancer. Radioresistance markedly impairs the efficacy of tumor radiotherapy and may involve antiapoptotic signal transduction pathways that prevent radiation-induced cell death. A common cellular response to genotoxic stress induced by radiation is the activation of the NF-κB pathway, which is heavily regulated by the UPS, as described above. The understanding of the mechanism by which UPS inhibition abolishes radiation-induced cell survival warrants further investigation and may lead to even more effective radiosensitizing agents affecting the UPS family. Furthermore, investigating the effects of proteasomes on important proliferative mediators such as p16, p21, p53, cyclin D, and others might not only lead to a better understanding of the mechanisms that contribute to resistance to radiation, but more importantly, might lead to more efficacious treatment strategies for advanced rectal cancer than we have today.

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