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THE PROTEASOME AND PROTEASOME INHIBITORS IN CANCER THERAPY

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■ **Abstract** The proteasome, a multicatalytic proteinase complex, is responsible for the majority of intracellular protein degradation. Pharmacologic inhibitors of the proteasome possess in vitro and in vivo antitumor activity, and bortezomib, the first such agent to undergo clinical testing, has significant efficacy against multiple myeloma and non-Hodgkin lymphoma (NHL). Preclinical studies demonstrate that proteasome inhibition potentiates the activity of other cancer therapeutics, in part by downregulating chemoresistance pathways. Early clinical studies of bortezomib-based combinations, showing encouraging activity, support this observation. Molecular characterization of resistance to proteasome inhibitors has revealed novel therapeutic targets for sensitizing malignancies to these agents, such as the heat shock pathway. Below, we review the pharmacologic, preclinical, and clinical data that have paved the way for the use of proteasome inhibitors for cancer therapy; outline strategies aimed at enhancing the efficacy of proteasome inhibitors; and review other potential targets in the ubiquitin proteasome pathway for the treatment of cancer.

INTRODUCTION

Proteolysis Through the Ubiquitin-Proteasome Pathway

Degradation of potentially toxic oxidized and misfolded proteins and controlled proteolysis of regulatory proteins, such as those involved in cell cycle progression and apoptosis, are essential for cell homeostasis. These processes are mediated primarily by the ubiquitin-proteasome pathway (UPP), which consists of the ubiquitin-conjugating system and the proteasome. The ubiquitin-conjugating system targets proteins for degradation via the sequential attachment of an

ubiquitin (Ub)* peptide to an E1 Ub-activating enzyme, the transfer of the activated Ub moiety to an E2 Ub-conjugating enzyme, and finally, covalent attachment of Ub to the target protein through the concerted action of an E2 and E3 Ub-ligase (1) (Figure 1). Over 500 E3s exist in the genome, each of which has the ability to interact with a limited number of substrates, thus providing specificity to proteasome-mediated proteolysis. Continued processing through the Ub-conjugating system generates a polyubiquitinated protein that serves as a substrate for destruction by the proteasome. Proteins can undergo monoubiquitination as well, a posttranslational modification that does not impact protein turnover. Monoubiquitination plays an important role in epigenetic control of gene transcription via histone modification and in trafficking of proteins in cells (2).

The proteolytic activities of the proteasome are located within a 20S multisubunit structure consisting of four stacked rings arranged around an inner catalytic chamber (3, 4). Each of the outer rings consists of seven polypeptide α subunits that serve as the gates through which proteasome substrates enter, whereas the two inner rings each consist of seven β subunits. The β-1, -2, and -5 subunits contain the postglutamyl peptidyl hydrolytic-like, tryptic-like, and chymotryptic-like proteolytic activities of the proteasome, respectively. Mammalian proteasomes are also capable of two other activities characterized by proteolysis after branched chain and small neutral amino acids (5). When cells are exposed to interferon-γ (IFN-γ), the β-1, -2, and -5 subunits are replaced by low-molecular-weight protein (LMP) subunits LMP-2, -10, and -7, respectively, which preferentially cleave substrates after hydrophobic and basic residues (6, 7). The LMP-containing 20S proteasome has been dubbed the immunoproteasome owing to its ability to generate peptides that may be preferred substrates for major histocompatibility complex (MHC) class I antigen presentation (8). Although immunoproteasomes are often thought of as strictly inducible structures, they are constitutively present in lymphoid tissues, such as the spleen and thymus (9, 10). Currently available proteasome inhibitors interfere with the chymotryptic-like activity of the 20S proteasome and immunoproteasome. Whether selective inhibition of either structure would change the therapeutic index of proteasome inhibitors is not known but worthy of further investigation, particularly given the significant antitumor activity of currently available nonselective proteasome inhibitors in tumors of lymphoid origin.

*Abbreviations: Cks, cdc kinase subunit; CDC, cell division control protein; CLL, chronic lymphocytic leukemia; CR, complete response; DLTs, dose-limiting toxicities; ER, endoplasmic reticulum; HSF, heat shock transcription factor; HSP, heat shock protein; IκB, inhibitor kappa B; JNK, c-Jun-N-terminal kinase; LMP, low-molecular-weight protein; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; MKP, mitogen-activated protein kinase phosphatase; MTD, maximum tolerated dose; NHL, non-Hodgkin lymphoma; NF-κB, nuclear factor kappa B; ORR, overall response rate; PA, proteasome activator; pegLD, pegylated liposomal doxorubicin; PR, partial response; siRNA, small interfering RNA; Skp, S-phase kinase-associated protein; TTP, time to progression; Ub, ubiquitin; UPP, ubiquitin proteasome pathway; UPR, unfolded protein response; VEGF, vascular endothelial growth factor.

The 20S proteasome requires the assembly of an activator, or regulatory complex, on the end of each outer ring of the 20S particle to process most proteins. The best-characterized proteasome activator is PA700 (proteasome activator 700 kilodaltons), also dubbed the 19S regulatory complex (11), which consists of a lid and a base. The lid contains at least nine polypeptide subunits, including isopeptidases that remove the polyubiquitin chain from the substrate in an ATP-dependent manner, allowing the release of free ubiquitin to be reused in further rounds of proteolysis. The base directly associates with the α rings and contains eight polypeptides, including six ATPases, which function to bind and unfold the substrate, open the channel of the 20S particle, and catalyze substrate translocation into the 20S proteasome. When capped by the 19S regulatory complex at each end, the 20S complex forms the core of the 26S proteasome (11).

Two other proteasome activators have been identified, PA28 (also known as 11S) and PA200, which bind to and activate the proteolytic functions of the proteasome (12). Two isoforms of PA28, α and β, form a heptameric complex that associates with the outer α rings of the 20S proteasome. PA28-α and -β are strongly upregulated upon exposure of cells to IFN-γ and associate with the 20S core particle (13, 14), which would suggest that they play an important role in generating peptide antigens for presentation in the context of class I MHC. However, mice lacking both α and β subunits have normal cellular immunity and assemble immunoproteasomes normally, although they are unable to process a specific epitope from the melanoma antigen TRP-2 (15). Therefore, PA28-α and -β may not be necessary for immunoproteasome assembly, but they are necessary for the class I MHC presentation of select epitopes. Hybrid proteasomes have been identified in which the 20S particle is capped by an 11S complex at one end and a 19S at the other, and they may play a role in antigen presentation as well (16, 17). PA200 is the most recently discovered proteasome activator and may be involved in DNA repair (18). Further elucidation of the biologic functions of different proteasome activators may pave the way for the development of other potential therapeutic targets in the UPP.

The Development of Proteasome Inhibitors

Interest in the function of the proteasome in normal cellular processes and human diseases paved the way for the development of proteasome inhibitors. The peptide aldehydes, the first class of compounds shown to inhibit the proteasome (19), are reversible serine and cysteine protease inhibitors that bind to the N-terminal active site threonine of proteolytically active proteasome subunits (Figure 2*A*). Initial attempts at synthesizing more specific proteasome inhibitors involved modification of the peptide moieties of the peptide aldehydes. However, crossreactivity with other proteases, including calpains and cathepsins, and excessive reactivity of the aldehyde pharmacophore limited the clinical potential of this class of compounds. In an attempt to improve the selectivity of agents toward the proteasome, Adams et al. (20) replaced the pharmacophore of peptide aldehydes with a boronic

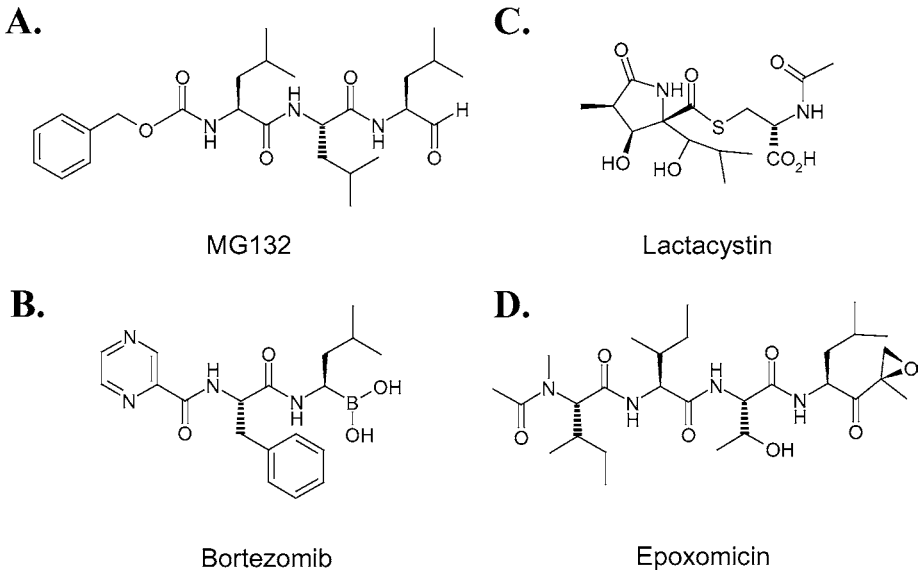

Figure 2 Representative examples of various classes of proteasome inhibitors. (*A*) MG-132, a tripeptidyl aldehyde compound, binds to and inhibits the active site of the 20S proteasome via the formation of a reversible hemiacetal adduct between the aldehyde group of MG132 and the hydroxyl group of the amino-terminal catalytic threonine. (*B*) Bortezomib is a dipeptidyl boronic acid that inhibits the proteasome via the formation of a reversible pseudotetrahedral complex between the amino-terminal catalytic threonine and the boronic acid pharmacophore. (*C*) Lactacystin, a β-lactone, irreversibly inhibits the active site of the 20S proteasome through the formation of an ester bond with the amino-terminal catalytic threonine. (*D*) Epoxomicin is an α',β'-epoxyketone that irreversibly inhibits the 20S proteasome via the formation of a morpholino ring between the amino-terminal catalytic threonine and the α',β'-epoxyketone pharmacophore of epoxomicin.

acid functional group, thus generating a series of peptide boronic acid inhibitors (Figure 2*B*). The best characterized of their inhibitors, bortezomib (VELCADE ®, also previously known as PS-341; Millennium Pharmaceuticals, Inc.), is a dipeptidyl boronic acid that reversibly inhibits the chymotrypsin-like activity of the proteasome. This agent displayed remarkable selectivity toward the proteasome relative to serine and cysteine proteases, and it possessed unique antitumor properties in a National Cancer Institute (NCI) tumor cell line screen (21). Bortezomib is the first proteasome inhibitor to have undergone clinical testing in patients, and it has demonstrated activity, particularly in multiple myeloma and non-Hodgkin lymphoma (NHL), as outlined below.

Nature has provided a wealth of other compounds that also inhibit the proteasome. Lactacystin was isolated from *Streptomyces lactacystinaeus*, and it initially drew interest based on its ability to induce differentiation of neuroblastoma cells

and to inhibit cell cycle progression (22, 23). It was later shown that lactacystin irreversibly inhibits the proteasome via a covalent modification of the amino-terminal threonine nucleophile of the beta subunits (24) (Figure 2*C*). An analog of the active metabolite of lactacystin, *clasto*-lactacystin β-lactone, has been successfully synthesized and tested in a phase I clinical trial (25). Hanada et al. (26) isolated epoxomicin, a peptide epoxyketone with activity against the B16 murine melanoma tumor model, from an Actinomycete strain. Epoxomicin has since been shown to selectively and irreversibly inhibit the proteasome (27), and its successful synthesis has led to the development of other peptide α',β'-epoxyketones that inhibit the chymotrypsin-like activity of the proteasome and that also possess antiproliferative and anti-inflammatory properties (28) (Figure 2*D*). Whether irreversible proteasome inhibitors such as the beta-lactones and epoxyketones will possess increased activity and/or toxicity compared with the current clinically available reversible inhibitor, bortezomib, is not yet known.

Most of the currently available proteasome inhibitors preferentially block the chymotrypsin-like activity of the proteasome, which is the rate-limiting proteolytic step (29). Selective blockade of other proteolytic activities of the proteasome, or simultaneous inhibition of several proteolytic activities, could alter the activity or toxicity profile of proteasome inhibitors. Initial support for this possibility comes from a recent report presented in abstract format investigating the use of NPI-0052, a novel proteasome inhibitor isolated from the fermentation broth of a marine Actinomycete with activity against the chymotryptic-, tryptic-, and postglutamyl peptidyl hydrolytic-like activities of the proteasome in multiple myeloma cell lines. Perhaps surprisingly, NPI-0052 induced apoptosis in myeloma cells that were resistant to bortezomib, and in combination, NPI-0052 and bortezomib led to synergistic induction of cell death (30). Future preclinical and clinical studies investigating the antitumor properties of the different classes of proteasome inhibitors and the molecular basis for their action are eagerly awaited.

Proteasome Inhibitors as Antitumor Agents

The discovery that the proteasome inhibitor lactacystin could cause cell cycle arrest led investigators to examine whether it can also induce apoptosis. Imajoh-Ohmi et al. (31) provided the first evidence that proteasome inhibitors possess antitumor properties by showing that treatment of the U937 monoblast cell line with lactacystin induced DNA fragmentation and morphologic changes consistent with apoptosis. Fujita et al. (32) demonstrated that proteasome inhibitors can sensitize cells to the apoptotic effects of other agents by showing that treatment of U937 cells with lactacystin and peptide aldehydes potentiated tumor necrosis factor–mediated cell death. Shortly thereafter, a peptide aldehyde proteasome inhibitor was shown to induce tumor growth delay in a murine xenograft model of Burkitt lymphoma, providing the first in vivo evidence of the antitumor activity of proteasome inhibitors (33). More recently, bortezomib was shown to have broad antitumor activity in an NCI tumor cell line screen and in several murine xenograft

models (21, 34, 35). These early observations have led to an explosion in preclinical and clinical research evaluating the role of proteasome inhibition in cancer therapy.

PROTEASOME INHIBITORS' PREFERENTIAL ACTIVITY AGAINST TRANSFORMED CELLS

Preclinical studies have repeatedly demonstrated a selective susceptibility of transformed cells to proteasome inhibition (33, 36–39). Several groups have explored the molecular basis by which proteasome inhibitors selectively mediate apoptosis in malignant cells and found that actively proliferating cells were more susceptible to proteasome inhibitor-mediated apoptosis than quiescent cells (40, 41). However, pheochromocytoma cells undergo apoptosis regardless of whether they are proliferating or have undergone differentiation (41), and preclinical tumor models, such as chronic lymphocytic leukemia (CLL), in which cells are predominantly in the G_0 phase of the cell cycle, remain susceptible to proteasome inhibitor-mediated apoptosis (42). Thus, the proliferative status of a cell does not fully explain increased sensitivity to proteasome inhibitors.

Recent evidence has linked upregulation of various UPP functions to the process of malignant transformation, which may explain the increased susceptibility of transformed cells to proteasome inhibition. CLL cells have threefold higher levels of chymotrypsin-like proteasome activity than normal lymphocytes (43). Low levels of the cyclin-dependent kinase inhibitor p27 are found in a large number of tumor types, including colon cancer and mantle cell lymphoma, and are associated with a poor prognosis. Importantly, low levels of p27 in malignant cells are often the result of increased proteasome-mediated degradation (44, 45) and/or upregulation of SCF^{Skp2}, a Ub ligase that targets p27 for degradation by the proteasome (46, 47). Similarly, using tissue samples of human prostate adenocarcinoma, Li & Dou (48) demonstrated that low levels of the pro-apoptotic Bcl-2 family member, Bax, are associated with increased proteasome-mediated degradation and more aggressive disease.

Dysregulation of UPP function has also been identified in multiple myeloma. Comparing the malignant plasma cells of a myeloma patient with the normal plasma cells of a genetically identical twin, Munshi et al. (49) demonstrated significant upregulation of a large number of UPP transcripts, including UbC, UbB, Ub-specific protease, proteasome subunit α, and Ub-activating E1-like enzyme. Gene expression profiling of a large number of patient-derived myeloma cells revealed that POH1 (or Rpn11) is upregulated in multiple myeloma and associated with a poor prognosis (50). POH1 is a deubiquitinating enzyme that is a component of the lid of the 19S regulator. POH1 is essential to the proteolytic properties of the proteasome and, therefore, has generated interest as a potential novel chemotherapeutic target in the UPP (47, 51). Interestingly, POH1 overexpression confers resistance to several chemotherapeutics, including doxorubicin (52, 53). More recently, investigators showed that Cks1b is overexpressed in a subset of myeloma patients and associated with a short survival (54). Cks1b is a cofactor that is essential for

the activity of the Ub ligase, Skp2 (55). The same investigators, as well as others, have found that expression of the Ub-conjugating enzyme, CDC34, is increased in myeloma samples relative to normal plasma cells (49, 50, 56). CDC34 forms part of an E2/E3 complex responsible for ubiquitination of a number of important substrates, including p27. Interestingly, downregulation of CDC34 in multiple myeloma cells potentiated the apoptotic activity of the proteasome inhibitor, bortezomib (56), which perhaps is unexpected given that concurrent inhibition of CDC34 and the proteasome would seem redundant. Regardless, the results suggest that factors upstream of the proteasome in the UPP are viable chemotherapeutic targets. In particular, pharmacologic inhibition of E3/substrate interactions, such as Skp2 and p27, may prove to be invaluable targets for future cancer therapies with increased selectivity and decreased toxicity relative to currently available proteasome inhibitors (47).

THE MOLECULAR SEQUELAE OF PROTEASOME INHIBITION

The majority of intracellular protein degradation occurs through the UPP (57). Consequently, proteasome inhibitors affect many cellular processes and likely achieve their antitumor effect through the modulation of a number of important pathways. Below, we outline some of the better-characterized targets of proteasome inhibitors.

Proteasome inhibitors first drew clinical interest as potential antineoplastic agents owing to their ability to downregulate the nuclear factor-kappa B (NF-κB) pathway. The NF-κB transcription factor family plays an important role in tumorigenesis via the transactivation of genes involved in cell proliferation, apoptosis, tumor cell invasiveness and metastasis, and angiogenesis (58). NF-κB is normally sequestered in the cytoplasm through association with its inhibitor, IκB. A diverse array of signals can lead to the phosphorylation of critical serine residues on IκB, targeting IκB for polyubiquitination and proteolysis by the UPP, which then allows NF-κB to translocate to the nucleus and mediate transcription (see Figure 1). Proteasome inhibitors block the degradation of IκB, thereby sequestering NF-κB in the cytoplasm and downregulating its transcriptional activity (59, 60). A salient example can be found in myeloma models. NF-κB was constitutively active in myeloma cell lines and patient myeloma samples (61, 62). Treatment of myeloma cell lines and patient samples with bortezomib resulted in growth inhibition, even in drug-resistant cell lines, and correlated with IκB stabilization and decreased NF-κB activity. Interestingly, bone marrow stroma-mediated production of interleukin-6, a cytokine that plays an important role in myelomagenesis, and whose transcription is upregulated by NF-κB, was attenuated by bortezomib, demonstrating that proteasome inhibitors may achieve their antimyeloma affects in part through modulation of the bone marrow microenvironment (38). In a murine xenograft model of head and neck squamous cell carcinoma, bortezomib inhibited tumor growth and angiogenesis, which correlated with downregulation of NF-κB

activity and decreased transcription of two proangiogenic factors, growth-regulated oncogene α and vascular endothelial growth factor, or VEGF (63).

NF-κB inhibition does not account for all of the antitumor effect of proteasome inhibitors. Treatment of myeloma cells with an IκB kinase inhibitor downregulated NF-κB activity to a similar degree as bortezomib but was not as effective at inhibiting cell proliferation (64). In other studies, treatment of CLL cells, solid tumor cell lines, and lymphoma cells with proteasome inhibitors induced apoptosis but did not lead to NF-κB inhibition (43, 65, 66). Given the broad range of proteasome substrates, it seems likely that other factors are involved in proteasome inhibitor-mediated apoptosis.

Several studies have implicated the c-Jun N-terminal kinase (JNK) pathway as an important factor in proteasome inhibitor-mediated apoptosis. Activation of JNK by various stressors induces mitochondrial release of cytochrome *c*, in part by promoting translocation of the proapoptotic Bcl-2 family member, Bax, to mitochondria through phosphorylation of 14-3-3 proteins (67, 68). Proteasome inhibition induced sustained activation of JNK in several model systems, and JNK inhibition abrogated proteasome inhibitor-mediated apoptosis (69, 70).

Bcl-2 family members play a critical role in the regulation of apoptosis and are substrates of the proteasome (71). A number of studies have implicated a role in the accumulation of proapoptotic Bcl-2 family members in proteasome inhibitor-induced apoptosis. The proapoptotic cleavage product of Bid, tBid, is degraded by the proteasome. Treatment of HeLa cells with the proteasome inhibitor MG-132, or mutation of Ub acceptor sites on tBid, led to accumulation of tBid and sensitized the cells to TNF-α- and Fas-induced apoptosis (72). Nikrad et al. (73) have evaluated the expression of a panel of Bcl-2 family members in cancer cell lines before and after proteasome inhibition and found that levels of the proapoptotic Bcl-2 family members Bik and/or Bim increased owing to inhibition of their proteasome-mediated degradation, whereas levels of Bid, Bax, Bcl-2, and Bcl-x_L were unaffected. Downregulation of Bik and Bim using small interfering RNA (siRNA) or treatment of Bik-/Bim double knockout mouse embryonic fibroblasts abrogated proteasome inhibitor-mediated apoptosis. Similarly, Zhu et al. (65) demonstrated rapid upregulation of Bik upon proteasome inhibition in cancer cell lines but no change in levels of Bak, Bax, Bcl-2, or Bcl-x_L. Downregulation of Bik using siRNA blocked the apoptotic action of proteasome inhibition. Bax interacts with and inhibits the antiapoptotic proteins Bcl-2 and Bcl-x_L in mitochondria, leading to cytochrome *c* release and activation of the caspase cascade. Treatment of Bcl-2-overexpressing Jurkat cells with lactacystin induced apoptosis, which correlated with accumulation of mitochondrial-associated Bax (48). Other studies have shown that bortezomib can induce phosphorylation and cleavage of Bcl-2 in association with G_2-M phase cell cycle arrest (74). Thus, proteasome inhibitors may block Bcl-2 activity by decreasing its transcription by NF-κB, by upregulating translocation of Bax to the mitochondria, or by increasing its proteolytic inactivation.

The tumor suppressor p53 is also a substrate of the proteasome, and many studies have demonstrated upregulation of p53 or its targets by proteasome inhibitors. Evidence that p53 plays a direct role in proteasome inhibitor-mediated apoptosis came

from Lopes et al. (41), who showed that expression of a dominant-negative p53 in Rat-1 fibroblasts attenuated proteasome inhibitor-induced apoptosis. However, other studies have shown that proteasome inhibitors activate apoptosis regardless of the p53 status of a cell (42).

p27, a proteasome substrate, leads to G_1-S phase cell cycle arrest by binding to and inactivating cyclin-dependent kinase complexes. Downregulation of p27 activity plays an important role in a number of tumor types as outlined above (44–47). Increased levels of p27 have been demonstrated upon proteasome inhibition in several studies (38). Direct support for a role of p27 accumulation in proteasome inhibitor-mediated apoptosis came from Kudo et al. (75), who showed that introduction of antisense p27 oligonucleotides into an oral squamous cancer cell line partially blocked apoptosis by proteasome inhibitors.

The endoplasmic reticulum (ER) has received attention recently as an organelle that can initiate apoptotic pathways. Analogous to the heat shock response, excessive accumulation of misfolded proteins in the ER leads to the induction of the unfolded protein response (UPR), which serves to alleviate ER stress. When compensatory mechanisms fail, ER-mediated apoptosis ensues (76). The proteasome plays a central role in not only nuclear and cytosolic protein degradation, but also in the proteolysis of misfolded proteins in the ER. Not surprisingly, proteasome inhibitors have been shown to induce ER stress, as shown by their ability to upregulate important mediators of the UPR (77, 78). Interestingly, unlike other ER stressors, proteasome inhibitors simultaneously downregulate the UPR by inhibiting the activation of the ER endoribonuclease/kinase, IRE1α, an enzyme responsible for the generation of the active spliced mRNA species of XBP-1, a transcription factor that activates transcription of genes crucial to the UPR. Combined treatment of myeloma cells with the proteasome inhibitor MG-132 and an ER stressor, tunicamycin, a compound that inhibits N-linked glycosylation in the ER, led to synergistic induction of cell death owing to the ability of MG-132 to dampen the UPR to tunicamycin (78). Given the fact that plasma cells are professional immunoglobulin-secreting cells, it is not surprising that an intact UPR is critical for myeloma cell homeostasis. In fact, terminal B cell differentiation into plasma cells requires the presence of XBP-1 (79). It is tempting to speculate that increased susceptibility of plasma cells to ER stress may in part explain why proteasome inhibitors have such dramatic clinical activity in myeloma relative to other tumor types.

PROTEASOME INHIBITORS SENSITIZE MALIGNANT CELLS TO STANDARD THERAPEUTICS

Proteasome inhibitors increase sensitivity of tumor cells to a number of conventional therapeutics by downregulating innate, inducible, and acquired resistance pathways. The NF-κB pathway plays an important role in inducible chemoresistance, whereby proapoptotic stimuli also activate antiapoptotic survival pathways (80). Therefore, proteasome inhibitors may enhance apoptosis of cancer cells to

other agents by downregulating NF-κB. The combination of bortezomib and CPT-11 led to synergistic induction of apoptosis in a colon cancer xenograft model, and treatment with CPT-11 alone activated NF-κB, whereas the combination therapy led to complete NF-κB inhibition (81). Similarly, the combination of bortezomib and radiation led to synergistic induction of cell death in a mouse colon cancer xenograft model (82). Treatment of myeloma cell lines resistant to melphalan, mitoxantrone, and doxorubicin with a subtoxic dose of bortezomib sensitized them to treatment with these agents (62). NF-κB activity in these resistant cell lines was higher than that of nonresistant myeloma cell lines, and, whereas treatment with anthracyclines induced NF-κB, bortezomib suppressed it.

Proteasome inhibitors may sensitize tumor cells to DNA-damaging agents by inhibiting the transcription of genes involved in DNA repair. Subtoxic concentrations of bortezomib sensitized chemotherapy-resistant myeloma cell lines and patient samples to doxorubicin and melphalan. Gene expression profiling demonstrated downregulation of transcripts involved in DNA repair, suggesting that abrogation of resistance to melphalan and doxorubicin was mediated in part by attenuating the protective response to genotoxic stress (83). Using an ovarian cancer cell line model, Mimnaugh et al. (84) have shown that proteasome inhibitors increased cisplatin-DNA adduct formation, blocked nucleotide excision repair of cisplatin-DNA adducts, prevented cisplatin-induced transcriptional upregulation of the excision nuclease ERCC-1, and potentiated cisplatin-mediated apoptosis.

P-glycoprotein (P-gp) is a cell surface transporter that functions as an efflux pump for intracellular toxins, including several classes of drugs, such as the anthracyclines, Vinca alkaloids, and epipodophyllotoxins. Overexpression of P-gp on the surface of cancer cells plays an important role in chemoresistance to these agents (85). Interestingly, proteasome inhibition led to accumulation of immature P-gp that is incapable of transporting drugs out of cells efficiently (86). Moreover, the gene for P-gp, MDR1, has been shown to be a target of the transcription factor, NF-κB (87). Thus, although it has not been tested directly, proteasome inhibitors may abrogate chemoresistance by interfering with the transcription and posttranslational maturation of P-gp.

The function of proteasome inhibitors as chemosensitizers is clouded by several observations. First of all, proteasome inhibition has not overcome chemoresistance in all cases. For example, bortezomib was unable to reverse breast cancer cell resistance to cyclophosphamide or cisplatin in vitro (34). Secondly, the sequence in which the drugs are administered may be important. Treatment of myeloma cells with the anthracycline doxorubicin followed by bortezomib led to a stronger synergistic induction of cell death compared to treatment using the reverse sequence (83). Treatment with gemcitabine and carboplatin concurrently with, or followed by, bortezomib induced apoptosis in a synergistic manner in vitro and in a mouse xenograft model of lung adenocarcinoma, whereas treatment with bortezomib followed by gemcitabine and carboplatin abrogated cell death (88). Similarly, gemcitabine followed by bortezomib was more effective at inducing apoptosis in pancreatic cancer cells than the reverse order (89). These findings

point to the need for additional elucidation of the mechanisms involved in the action of these agents, so that their optimal use in patients can be identified.

ABROGATING INDUCIBLE AND DE NOVO CHEMORESISTANCE TO PROTEASOME INHIBITORS

Although proteasome inhibitors have received a great deal of attention owing to their ability to attenuate inducible resistance to standard chemotherapeutics, it is becoming increasingly clear that they can activate antiapoptotic as well as proapoptotic pathways, thus attenuating their antitumor efficacy. The family of heat shock proteins (HSPs) plays an important role in mediating resistance to apoptosis (90, 91), and proteasome inhibition leads to a marked upregulation of HSPs, most notably HSP-72. Zhou et al. (92) showed that tripeptidyl aldehyde proteasome inhibitors and lactacystin dramatically upregulated expression of HSP-72. Bush et al. (93) also demonstrated induction of HSP-72 upon proteasome inhibition. Incubation of cells with proteasome inhibitors for a short period of time protected them from heat shock–induced cell death, revealing for the first time that proteasome inhibitors might be exploited therapeutically as inhibitors of apoptosis. Meriin et al. (69) demonstrated that sustained treatment of cancer cell lines with MG-132 led to the induction of apoptosis and correlated with activation of JNK and upregulation of HSP-72. An antiapoptotic function of proteasome inhibitors was also uncovered by a short incubation of cells with MG-132. Under these conditions, HSP-72 expression increased in cells, suppressed JNK activation, and prevented the induction of JNK-mediated apoptosis by subsequent heat stress and ethanol treatment. Robertson et al. (94) were the first to demonstrate the feasibility of HSP-72 blockade as a means of abrogating inducible chemoresistance to proteasome inhibitors. They were able to show that introduction of HSP-72 antisense oligonucleotides potentiated the proapoptotic activity of the proteasome inhibitor, MG-132. Gabai et al. (95) more recently have shown significant potentiation of apoptosis of prostate cancer cells to MG-132 through the downregulation of HSP-72 using siRNA and antisense oligonucleotide techniques. Interestingly, baseline HSP-72 expression was markedly upregulated in the malignant plasma cells of a myeloma patient compared with normal plasma cells from a genetically identical twin, suggesting that increased HSP-72 expression may play a role in de novo as well as inducible chemoresistance to proteasome inhibitors (49).

Other members of the HSP family likely play a role in inducible resistance to proteasome inhibitors. Gene transcriptional profiling of myeloma cells treated with bortezomib revealed that multiple members of the heat shock family are induced by proteasome inhibition, including HSP-90 (96). Treatment of myeloma cells with the combination of bortezomib and an HSP-90 inhibitor induced synergistic cell death. Similarly, Mimnaugh et al. (97) showed that treatment of breast cancer cell lines with the combination of a proteasome and HSP-90 inhibitor was more effective at inducing apoptosis than either agent alone, and that the combination

regimen was associated with a dramatic increase in intracellular ubiquitinated protein aggregates. Characterization of the gene expression profile of the proteasome inhibitor-sensitive DHL6 and -resistant DHL4 lymphoma cell lines demonstrated that HSP-27 is upregulated in resistant DHL4 cells. Downregulation of HSP-27 in DHL4 cells using an antisense strategy restored bortezomib sensitivity. Conversely, overexpression of HSP-27 in the sensitive DHL6 cell line rendered it resistant to bortezomib (98). Clearly, the development of pharmacologic inhibitors of the heat shock response as a means of abrogating de novo and inducible resistance to proteasome inhibitors represents a fruitful avenue for future research.

We have previously shown that proteasome inhibitors upregulate the mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1 at the transcriptional level (99). Like the family of HSPs, MKP-1 is potently induced by a variety of stressors, including arsenite, UV light, heat shock, and hydrogen peroxide (100). MKP-1 can dephosphorylate and deactivate JNK, an important mediator of proteasome inhibitor-induced apoptosis. We hypothesized that induction of MKP-1 represents a form of inducible resistance to proteasome inhibitors by attenuating maximal activation of JNK. Treatment of breast cancer cell lines overexpressing MKP-1 decreased proteasome inhibitor-mediated apoptosis. Conversely, cells in which MKP-1 was downregulated through siRNA techniques, and mouse embryonic fibroblasts lacking MKP-1, were more susceptible to the apoptotic effect of proteasome inhibitors. MKP-1 suppression of apoptosis correlated with decreased phospho-JNK levels, whereas increased apoptosis owing to downregulation of MKP-1 was associated with increased phospho-JNK levels (101). Anthracyclines repress transcription of MKP-1 (102), suggesting that they may be able to abrogate inducible resistance to proteasome inhibitors. Addition of anthracycline to bortezomib treatment led to the downregulation of bortezomib-mediated MKP-1 induction, strong activation of JNK, and enhanced induction of apoptosis. Infection of cells with an Adenovirus expressing MKP-1 under a promoter that is not modulated by anthracyclines attenuated induction of apoptosis by the combination regimen, and similar results were seen in a mouse xenograft model (101). These results further support the rationale for combining proteasome inhibitors and anthracyclines in the clinic, and for the development of MKP-1 inhibitors for clinical use.

CLINICAL STUDIES OF THE PROTEASOME INHIBITOR BORTEZOMIB

Pharmacology

Animal studies indicated that bortezomib injected intravenously leaves the vascular compartment within minutes, rendering correlations between plasma concentrations and proteasome inhibition, drug toxicity, and clinical activity difficult. To overcome this hurdle, Lightcap et al. (103) developed a pharmacodynamic assay for determination of proteasome inhibition in whole blood that has been used in clinical trials to guide dosing. After intravenous injection, bortezomib is widely

distributed in tissues but does not gain access to privileged sites such as the brain, spinal cord, eyes, and testes. Only recently has the metabolism of this agent been characterized. The primary route of metabolism in humans is oxidative deboronation, which leads to the production of two inactive diastereomeric carbinolamide metabolites. Several cytochrome P450 isoforms are capable of mediating the generation of the carbinolamide metabolites in vitro. Bortezomib then undergoes secondary metabolism and is ultimately excreted in the bile and urine (21, 104). Importantly, preliminary observations in myeloma patients with baseline renal insufficiency (baseline creatinine clearance of <30 ml/min) suggest that bortezomib can be safely administered to this population of patients, with similar levels of proteasome inhibition 1 h after dosing and similar rates of proteasome recovery (105). The safety of bortezomib administration in patients with hepatic insufficiency has not been evaluated.

Single-Agent Studies

There have been several phase I clinical trials evaluating the use of bortezomib for the treatment of refractory and relapsed malignancies. Forty-three patients with solid tumors were treated with bortezomib on days 1, 4, 8, and 11 of a three-week cycle. Dose-limiting toxicities (DLTs) included diarrhea and sensory neuropathy. The gastrointestinal toxicities are interesting and perhaps not surprising given the fact that high levels of radiolabeled bortezomib accumulated in the small intestine of rats shortly after intravenous injection, and later in the large intestine (21). Pharmacodynamic studies showed a dose-dependent inhibition of proteasome function, with a mean proteasome inhibition of 68% one hour after the 1.56 mg/m^2 dose. Although a maximum tolerated dose (MTD) was not identified, the dose recommended for further study was 1.56 mg/m^2. One patient with nonsmall cell lung carcinoma had a partial response (PR) to therapy, whereas three other patients had stable disease as their best responses (106). In a second study, 53 patients with refractory solid tumors, 48 of whom had androgen-independent prostate cancer, were treated with escalating doses of bortezomib once weekly for four weeks on a five-week cycle. Using this dosing scheme, the MTD was 1.6 mg/m^2. Gastrointestinal toxicities such as diarrhea were particularly common and dose dependent. Pharmacokinetic analyses demonstrated a rapid initial distribution half-life of 0.22 to 0.46 h, a terminal half-life of greater than 10 h, and a volume of distribution greater than 500 liters, similar to the pharmacokinetics observed in animal studies. Not surprisingly, the correlation between plasma levels and degree of proteasome inhibition was poor. In contrast to other phase I studies, pharmacodynamic analysis of proteasome inhibition revealed that proteasome activity did not return completely to normal prior to the next dose of bortezomib, which may explain the increased incidence of toxicities with subsequent cycles of treatment. Tachyphylaxis or tolerance was not observed. Of the prostate cancer patients, two had a greater than 50% reduction in PSA levels, whereas two patients with measurable lymphadenopathy had PRs (107).

We undertook a phase I trial of bortezomib in patients with advanced hematologic malignancies. Patients received twice weekly bortezomib treatment for four consecutive weeks over a six-week cycle. Using this dosing scheme, the MTD was 1.04 mg/m^2 and DLTs included thrombocytopenia, hyponatremia, hypokalemia, fatigue, and malaise. Peripheral neuropathy was observed but manageable. Proteasome function was inhibited in a dose-dependent fashion with 74% inhibition achieved at the 1.38 mg/m^2 dose. Evidence of activity was seen in nine out of nine evaluable patients with plasma cell dyscrasias, including one patient with multiple myeloma who had a durable complete response (CR). PRs were seen in two patients with NHL, including one with mantle cell lymphoma and another with follicular lymphoma (108).

The encouraging preclinical and phase I activity of bortezomib in myeloma led to a phase II trial (the SUMMIT study) in which 202 heavily pretreated myeloma patients received 1.30 mg/m^2 of bortezomib on days 1, 4, 8, and 11 of a three-week cycle for up to eight cycles. CRs and near-CRs were seen in 10% of the patients. The overall response rate (ORR, or combined CR, near-CR, and PR rate) was 27%, and 59% of patients had stable disease or better. The median duration of response among patients with a CR, PR, or minor response was 12 months, and the median time to progression (TTP) for all of the patients was 6.6 months, more than twice the three-month progression time on whatever had been their previous therapy. Median overall survival was 16 months. These encouraging findings led to the approval of bortezomib by the Food and Drug Administration as therapy for patients with multiple myeloma that had been treated with at least two prior therapies and had progressed on the last of these. Grade 3 and 4 toxicities occurring in more than 10% of patients included fatigue, thrombocytopenia, neutropenia, and peripheral neuropathy. Eighteen percent of patients had to discontinue therapy owing to drug-related adverse events (109). A second phase II trial (the CREST study) in myeloma evaluated two doses of bortezomib, 1.0 and 1.3 mg/m^2, given twice weekly for two weeks on a three-week cycle. The ORR was 33% and 38% at the two dose levels, respectively. When dexamethasone was added to bortezomib for patients who had progressive disease after two cycles, or stable disease after four, the ORR was 37% and 62% at the two dose levels. Grade 3 and 4 adverse events included peripheral neuropathy, thrombocytopenia, weakness, neutropenia, pneumonia, and hyponatremia. Twelve of 54 patients discontinued therapy as a result of adverse events, 5 of which were due to neuropathy (110).

The peripheral neuropathy seen with bortezomib is intriguing, particularly given the fact that accumulation of misfolded proteins plays an important role in the pathogenesis of a variety of neurodegenerative diseases, such as Alzheimer's disease. The incidence of peripheral neuropathy seen in both of the phase II myeloma trials, SUMMIT and CREST, was 35%. However, 80% of the patients enrolled on these trials had preexisting neuropathy, and these patients were more likely to develop grade 3 neuropathy on bortezomib than patients with no antecedent neuropathy (16% versus 3%, respectively). Twelve percent of patients required dose reductions and 5% discontinued therapy owing to neuropathy. Nerve

conduction studies and quantitative sensory testing demonstrated that the neuropathy is a length-dependent, axonal sensory neuropathy predominantly affecting small fibers (111). Whether neuropathy is due to proteasome inhibition or an off-target effect is not known.

More recently, the final results of a large, phase III multicenter trial comparing the effectiveness of bortezomib versus dexamethasone for refractory myeloma were presented. The ORR was 38% and 18%, 1-year survival 80% and 66%, and median TTP 6.2 and 3.5 months, respectively, for the two therapies, all of which were statistically significant for the superiority of bortezomib. Grade 3 toxicities were seen more frequently in the bortezomib arm and included gastrointestinal side effects, thrombocytopenia, neutropenia, and peripheral neuropathy. The rates of grade 4 adverse events were similar between the two groups (112). Early results of a phase II study evaluating single-agent bortezomib in previously untreated myeloma patients were recently presented as well. Out of 22 evaluable patients, 5% and 36% of patients have achieved CRs and PRs, respectively, for an ORR of 41%. Only 2% of patients had progressive disease. Peripheral neuropathy occurred in 21% of subjects, which was mostly grade 2 in severity, but did require dose reduction in four patients and discontinuation of therapy in one patient with grade 3 neuropathy. This suggests that prior treatment with neurotoxic agents is not necessary for the manifestation of this toxicity (113).

Two phase II studies of bortezomib in NHL were recently published and demonstrated promising activity. O'Connor et al. (114) treated 26 patients with indolent NHL and mantle cell lymphoma at a dose of 1.5 mg/m^2 given twice weekly for two weeks on a three-week cycle. Of the 24 patients evaluable for response, 58% had a PR or better. Goy et al. (115) treated 60 patients with relapsed or refractory B cell NHL using an identical dose and treatment cycle. Twelve of 29 patients with mantle cell lymphoma had a response, including 6 CRs. Four of the 21 evaluable patients with other subtypes of NHL responded. In both studies, common toxicities included thrombocytopenia, fatigue, neutropenia, and peripheral neuropathy, most of which were grade 1 or 2 in severity. Given the encouraging results in mantle cell lymphoma and low-grade NHLs, clinical trials incorporating bortezomib into combination lymphoma regimens are currently underway.

Unfortunately, the clinical activity of bortezomib in other tumors has been less promising, with marginal or no responses in diseases such as renal cell carcinoma, neuroendocrine tumors, sarcomas, acute leukemias, and melanoma (116–121). Given the ability of proteasome inhibitors to sensitize malignant cells to a broad array of conventional chemotherapeutics, they may best be suited for combination strategies in these diseases.

Combination Studies

Given the strong preclinical rationale for combining anthracyclines with proteasome inhibitors, we conducted a phase I clinical trial evaluating the combination of

bortezomib and pegylated liposomal doxorubicin (pegLD; Doxil®, Tibotec Therapeutics) in patients with advanced hematologic malignancies. Subjects received pegLD at a fixed dose of 30 mg/m^2 on day 4, while bortezomib was given at escalating doses from 0.9 mg/m^2 to 1.5 mg/m^2 on days 1, 4, 8, and 11. Although an MTD was not identified, the recommended dose of bortezomib for further study was 1.3 mg/m^2 because frequent dose delays and reductions were needed at the higher dose levels. The number of subjects at the 1.3 mg/m^2 dose was expanded to gain further experience with the combination regimen. Grade 3 and 4 toxicities included fatigue, thrombocytopenia, neutropenia, peripheral neuropathy, diarrhea, and pneumonia. Out of 22 evaluable patients with myeloma, 8 achieved CRs or near-CRs, whereas another 8 achieved PRs, for an ORR of 73%. Only one patient with myeloma had progressive disease after completing the first two cycles of therapy. Thirteen of the myeloma patients had previously received anthracycline-based regimens and were reported to have progression, stable disease, or an initial response followed by stable or progressive disease. Of these patients, four had CRs, one a near-CR, and three had PRs, suggesting that proteasome inhibitors may indeed abrogate anthracycline-based drug resistance in vivo. A CR was also seen in a patient with multiply relapsed peripheral T cell lymphoma, and two of five patients with B-cell NHL had PRs (122). Given the striking activity of this regimen in myeloma, the Cancer and Leukemia Group B (CALGB) is currently conducting a phase II trial of pegLD and bortezomib in patients with newly diagnosed myeloma. A randomized phase III trial comparing bortezomib with the combination of bortezomib and pegLD in patients with relapsed or refractory myeloma is also underway.

Results of a phase I trial of bortezomib in combination with dexamethasone and doxorubicin for patients with newly diagnosed multiple myeloma were recently reported. Subjects received bortezomib at 1.3 mg/m^2 on days 1, 4, 8, and 11; dexamethasone at 40 mg on days 1–4, 8–11, and 15–18 on cycle 1 and on days 1–4 of cycles 2–4; and infusional doxorubicin on days 1–4 at 0, 4.5, and 9 mg/m^2/day of a 21-day cycle. Of the 21 patients treated, none experienced any DLTs. Two subjects had to discontinue therapy, one because of postural hypotension and the other because of peripheral neuropathy. Peripheral neuropathy was common and occurred in 45% of patients but was grade 1 in severity in all but one case. The ORR for all dose cohorts was an impressive 95%, with a CR rate of 25% and a near-CR rate of 4%. Importantly, the combination regimen did not appear to negatively impact the ability to mobilize stem cells from these patients (123).

Given the additive activity of bortezomib and dexamethasone in preclinical myeloma studies; the ability of bortezomib to downregulate production of IL-6, an important mediator of corticosteroid resistance in the bone marrow microenvironment; and the ability of dexamethasone to capture responses in some patients with stable or progressive disease on single-agent bortezomib in the above mentioned SUMMIT trial (109), Jagannath et al. (124) evaluated the efficacy of bortezomib alone and in combination with dexamethasome for patients with newly diagnosed myeloma. All 32 patients received single-agent bortezomib at 1.30 mg/m^2 on days

1, 4, 8, and 11 of a 3-week cycle for the first two cycles. The response rate after two cycles of treatment was 40%, with 12% combined CRs and near-CRs. Dexamethasone was added after cycle 2 if less than a PR was achieved and after cycle 4 if less than a CR was attained. In total, dexamethasone was added to therapy for 22 patients. The ORR in this study was 88%, with 6% CRs and 19% near-CRs.

Based on an index patient who had responded to the combination of bortezomib and thalidomide after progression on single-agent bortezomib and documented prior resistance to thalidomide, Zangari et al. (125) evaluated the safety and activity of the combination of thalidomide and bortezomib in a phase I/II trial for patients with refractory multiple myeloma. To date, 79 patients with advanced myeloma have been enrolled, 80% of whom had cytogenetic abnormalities and 80% of whom had received prior autologous stem cell transplants. PRs and near-CRs have been observed in 40% of this highly refractory patient population. Other therapies are currently being tested in patients with relapsed/refractory and newly diagnosed myeloma, including bortezomib in various combinations with dexamethasone, thalidomide, melphalan, and anthracyclines. Preliminary evidence of activity is quite promising. The final results of these trials are eagerly awaited, and hopefully will provide insight into the optimal way to incorporate bortezomib into current myeloma therapies.

Combination regimens in other tumor types, including mantle cell lymphoma, low-grade NHLs, and solid tumors, are actively being tested but results are less mature. To date, there have not been reports of any pharmacologic interactions with other agents.

CONCLUSIONS

The UPP affects a large number of cellular processes essential to tumorigenesis. Preclinical studies have demonstrated notable in vitro and in vivo antitumor activity of proteasome inhibitors. Upregulation of various UPP functions in the process of malignant transformation may in part explain the selectivity of these agents toward malignant cells. A better understanding of the role that deregulated UPP activity plays in various malignancies will likely lead to the development of drugs that target other components of the UPP. Studies have shown that proteasome inhibitors can abrogate many forms of drug resistance and synergize with a number of conventional therapies. However, proteasome inhibitors are also capable of activating antiapoptotic pathways that attenuate their antitumor activity. Preclinical work has shown that important mediators of the response to environmental stress, HSPs and MKP-1, play prominent roles. Strategies aimed at blocking the activation of the stress response to proteasome inhibitors may enhance their efficacy. Further molecular characterization of the basis of resistance to proteasome inhibitors will likely yield other potential targets for combination therapy. The clinical experience with the proteasome inhibitor bortezomib demonstrates tolerable

toxicities and significant activity in multiple myeloma and NHL. Early phase I data demonstrates remarkable activity of the combination of pegLD and bortezomib in myeloma, including in anthracycline unresponsive disease, suggesting that bortezomib can abrogate drug resistance in patients, as well. The results of currently active combination trials in patients with solid tumors and hematologic malignancies are eagerly awaited, but it seems safe to conclude that proteasome inhibitors will be an important part of our chemotherapeutic armamentarium against cancer. Future clinical studies of novel proteasome inhibitors and other agents that target the UPP are also likely to yield a wealth of new drugs for cancer patients.

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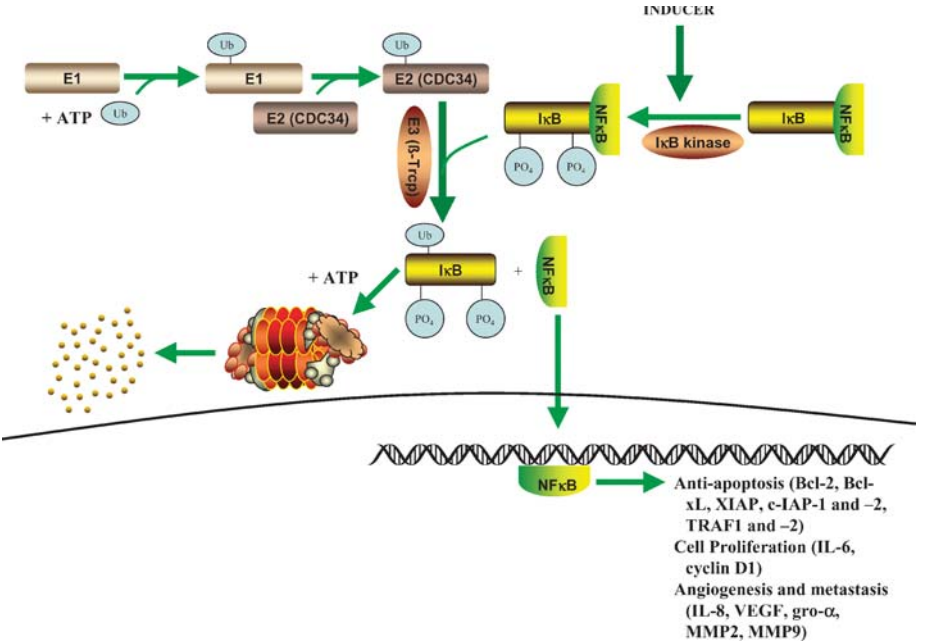

Figure 1 Control of the NF-κB pathway through the ubiquitin conjugating system. The transcription factor, NF-κB, is ordinarily sequestered in the cytoplasm through its interaction with IκB. A variety of stimuli (TNFα signaling, chemotherapeutics, radiation) lead to the phosphorylation of critical serine residues on IκB via the IκB kinase. Once phosphorylated, IκB is ubiquitinated and marked for degradation via the proteasome. Ubiquitination of IκB occurs via the sequential attachment of an Ub peptide to an E1 Ub-activating enzyme, the transfer of the activated Ub moiety to CDC34 (an E2 Ub-conjugating enzyme), and covalent attachment of ubiquitin to IκB through the concerted action of CDC34 and the E3 Ub-ligase, β-Trcp. Once IκB is degraded, NF-κB is free to translocate to the nucleus, where it mediates the transcription of genes crucial to cell survival, cell proliferation, angiogenesis and metastasis, and drug resistance [this figure has been adapted from ScienceSlides 2005 (VisiScience Inc.; http://www.visiscience.com) with permission of the publisher].

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