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Induction of Tumor Cell Apoptosis by a Proteasome Deubiquitinase Inhibitor Is Associated with Oxidative Stress

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Abstract

Aims: b-AP15 is a recently described inhibitor of the USP14/UCHL5 deubiquitinases (DUBs) of the 19S proteasome. Exposure to b-AP15 results in blocking of proteasome function and accumulation of polyubiquitinated protein substrates in cells. This novel mechanism of proteasome inhibition may potentially be exploited for cancer therapy, in particular for treatment of malignancies resistant to currently used proteasome inhibitors. The aim of the present study was to characterize the cellular response to b-AP15-mediated proteasome DUB inhibition. **Results:** We report that b-AP15 elicits a similar, but yet distinct, cellular response as the clinically used proteasome inhibitor bortezomib. b-AP15 induces a rapid apoptotic response, associated with enhanced induction of oxidative stress and rapid activation of Jun-N-terminal kinase 1/2 (JNK)/activating protein-1 signaling. Scavenging of reactive oxygen species and pharmacological inhibition of JNK reduced b-AP15-induced apoptosis. We further report that endoplasmic reticulum (ER) stress is induced by b-AP15 and is involved in apoptosis induction. In contrast to bortezomib, ER stress is associated with induction of α -subunit of eukaryotic initiation factor 2 phosphorylation. **Innovation:** The findings establish that different modes of proteasome inhibition result in distinct cellular responses, a finding of potential therapeutic importance. **Conclusion:** Our data show that enhanced oxidative stress and ER stress are major determinants of the strong apoptotic response elicited by the 19S DUB inhibitor b-AP15. *Antioxid. Redox Signal.* 21, 2271–2285.

Introduction

THE UBIQUITIN PROTEASOME SYSTEM (UPS) is the major intracellular protein degradation system in eukaryotic cells (23). Ubiquitin is a conserved 76-amino-acid protein that is covalently attached to target proteins *via* a series of enzymatic steps, resulting in the trafficking of tagged proteins to the 26S proteasome. The 26S proteasome complex functions as the cell's molecular shredder and consists of a proteolytic 20S core particle (20SCP) that is associated with one or two 19S regulatory particles (19SRPs) (23). The 19SRP binds polyubiquitin-tagged proteins and promotes substrate translocation into the catalytic chamber where protein degradation is mediated by the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing activities of the 20SCP (18).

The efficient degradation of ubiquitinated substrates requires both unfolding and removal of polyubiquitin chains.

Innovation

Although proteasome inhibitors are effective for treatment of multiple myeloma, resistance eventually develops. The small molecule b-AP15 inhibits proteasomal degradation by inhibiting the USP14/UCHL5 deubiquitinases of the 19S regulatory particle. Here, we show that b-AP15 induces a distinct cellular response compared with a 20S core particle inhibitor. The potent apoptotic activity of b-AP15 is associated with strong increases of chaperone expression and strong induction of oxidative stress. Further, we show that inhibition of PKR-like ER kinase activation during endoplasmic reticulum stress is not essential for the cytotoxicity of proteasome inhibitors. The finding that different modes of blocking proteasome function induce distinct cellular responses has therapeutic implications.

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The translocation of ubiquitin-tagged proteins into the proteolytic core is facilitated by the deubiquitinase (DUB) activity of the 19SRP. In the absence of deubiquitination, bulky ubiquitin moieties would impede entry of the substrate into the 20SCP chamber. DUBs cleave the isopeptide bonds between the C-terminal carboxyl of ubiquitin and the amino group of a lysine residue on an adjacent protein and salvage ubiquitin for reuse by the UPS (23). The 19SRPs of metazoan cells are associated with three different DUBs. USP14 and UCH37/UHL5 are cysteine isopeptidases that cleave distal polyubiquitin chains and are suggested to promote substrate rescue rather than degradation (33). The third DUB of the 19SRP is the RPN11/POH1 metalloprotease, a Zn^{2+} -dependent protease of the JAMM family that is localized within the lid of the 19SRP. RPN11/POH1 performs ubiquitin chain amputation by cleaving entire chains from substrates in a process that is tightly coupled to degradation (60,64).

Ubiquitinated proteins initially bind to the 19S subunits Rpn10 and Rpn13, but substrate entry requires interactions between polyubiquitin chain and the 19SRP DUBs (21,50). Binding of polyubiquitin chains to USP14 (Ubp6 in yeast) promotes opening of the gate to the 20SCP (50). It was recently shown that substrate deubiquitination is coupled to stimulation of the activities of the AAA + ATPases in the base of the 19SRP that unwind protein substrates (51). Thus, binding of polyubiquitin conjugates to yeast Ubp6, or to USP14 and UCH37 in mammalian cells, leads to stimulation of 19SRP ATPase activity.

The dipeptidyl boronic acid bortezomib (PS-341, Velcade[®]) is a selective inhibitor of the proteolytic activity of the 20SCP. Bortezomib has shown activity against several malignant cell types and has been approved by the US Food and Drug Administration as a first line treatment for patients with multiple myeloma (6). The downstream effects of proteasome inhibition that ultimately result in cell death are not completely understood. Proteasome inhibition generates a plethora of cellular responses, including inhibition of NF κ B signaling (24), stabilization of Myc (45) and deregulation of Myc signaling (7), induction of endoplasmic reticulum (ER) stress (15), production of reactive oxygen species (ROS) (15,36,49), and amino-acid depletion-induced autophagy (56).

A number of groups have reported that generation of cellular ROS is associated with proteasome inhibitor-induced cell death (15,36,49). The interpretation of some of these studies is complicated by later findings of direct complex formation between proteasome inhibitor scavengers such as

N-acetyl cysteine (NAC), vitamin C, edaravone, and Tiron (14,19,37,68). The role of oxidative stress in the induction of apoptosis and cell death is, therefore, somewhat controversial. The mechanism(s) of how ROS is induced in cells exposed to proteasome inhibitors are also unclear.

The ubiquitin system is believed to be highly druggable, but has been underexplored by drug developers (8). We recently identified b-AP15 as an inhibitor of the USP14 and UCHL5 DUBs of the 19SRP (10). Inhibition of both USP14 and UCHL5 leads to a functional block of the proteasome (32) and we, indeed, observed accumulation of various proteasomal substrates in cells exposed to b-AP15 (10). Since b-AP15 blocks the proteasome by a distinct mechanism to that of the clinically used agent bortezomib, it is conceivable that b-AP15 (or an optimized lead) could be used for treatment of multiple myeloma patients who have developed resistance to bortezomib. Our previous studies showed that b-AP15 and bortezomib differ with regard to the apoptotic responses that are elicited. We found that apoptosis by b-AP15, but not by bortezomib, was insensitive to the overexpression of Bcl-2 and genetic disruptions of *BAX* and *TP53* (10). These results were not expected from models in which apoptosis induction by proteasome inhibitors is due to stabilization of various proteasomal substrates such as I κ B (24,55), p53 (63), the proapoptotic proteins Bid and Bax (5,35), and Myc (45). To understand and characterize the mechanisms of b-AP15-induced cytotoxicity, we have delineated the various cellular stress pathways affected. We report that disruption of redox homeostasis and induction of high levels of ROS is a major difference between the cellular responses to b-AP15 and bortezomib.

Results

Proteasome inhibition by bortezomib and b-AP15 induces a similar stress response

Proteasome inhibitors show a unique cytotoxic profile in the National Cancer Institute 60 cell line panel (NCI₆₀) when compared with thousands of other compounds (1). We compared the cytotoxic profiles of bortezomib (NSC681239) and b-AP15 (NSC687852) in the NCI₆₀ panel, using data available in the NCI Developmental Therapeutics Program database (www.dtp.nci.nih.gov). A weak correlation of the sensitivities of the NCI₆₀ cell lines to the two drugs was observed (Fig. 1A). Drugs in the NCI database have been assembled into self-organizing maps based on their cytotoxicity profiles, where

FIG. 1. b-AP15 and bortezomib induce similar but not identical cellular responses. (A) The IC₅₀ values to b-AP15 (NSC687852) and bortezomib (NSC681239) of different cell lines in the NCI₆₀ cell line panel are plotted. Each dot represents the IC₅₀ values of the two drugs in one cell line. The result shows a weak, but statistically significant, correlation of the cytotoxic responses to the two drugs in the NCI₆₀ cell line panel. Statistical analysis: Pearson correlation coefficient. (B) Self-organizing map of the NCI₆₀ tumor cell line panel. Compounds segregate into six major response categories: mitosis (M); nucleic acid metabolism (S); metabolic stress and cell survival (Q); membrane function (N); kinases/phosphatases and oxidative stress (P); and four unexplored regions, R, F, J, and V (27). b-AP15 and bortezomib map to distinct regions of the Q-region. (C) mRNA abundance of 84 genes reported to respond to stress were quantified by real-time quantitative polymerase chain reaction. HCT116 cells were exposed to b-AP15 or bortezomib for 6 h, RNA was extracted, and mRNA levels were determined after reverse transcription. The yellow dotted line shows a y/x slope of 1; a y/x slope 1.67 was observed for transcript levels of cells exposed to b-AP15/bortezomib. (D) Expression of various classes of genes in HCT116 cells exposed to b-AP15 or bortezomib as fold induction (red dots = b-AP15; blue dots = bortezomib). (E, F) Expression of specific genes after exposure of HCT116 cells to b-AP15 or bortezomib (fold induction). Blue: HSF-1-regulated genes, green: genes associated with oxidative stress; red: genes associated with ER stress. IC₅₀, 50% inhibitory concentration; NCI, National Cancer Institute; HSF-1, heat shock factor protein 1.

the position of a particular drug reflects its main mechanism of action (52). The majority of clinically used cancer therapeutics map to the “M” and “S” areas, where “M” represents agents that interfere with mitosis and “S” represents agents which interfere with nucleic acid synthesis. Bortezomib and b-AP15 map to distinct subregions of the “Q” area, associated with metabolic stress (Fig. 1B). These results suggest that bortezomib and b-AP15 display related, yet distinct, mechanisms of action.

We previously reported that b-AP15 treatment induces a gene expression profile related to that of several characterized proteasome inhibitors (10). We extended this analysis to a subset of stress responsive genes that are commonly affected by chemotherapeutic reagents. Colon carcinoma cells were used, as these are known to be sensitive to b-AP15 (10). HCT116 colon carcinoma cells were exposed to b-AP15 or bortezomib at IC₉₀ concentrations, and gene expression was determined by quantitative polymerase chain reaction

(qPCR). Both drugs induced a similar pattern of expression of an 84 gene set (Spearman $r=0.80$, $p<0.0001$; Fig. 1C), with genes associated with oxidative stress, ER stress, heat shock, and the immediate early response displaying major alterations in gene expression (Fig. 1D). However, the amplitude of activation of several genes was higher in b-AP15-exposed cells (best-fit slope = 1.67). The most conspicuous difference in the response to b-AP15 and bortezomib was the >1000-fold induction of *HSPA6* by b-AP15 (Fig. 1E). *HSPA6* encodes the Hsp70B' protein, a heat-shock induced chaperone that acts as the cells' final defense against proteotoxicity (46). Genes regulated by heat shock factor-1, mostly chaperone genes, were in general more strongly induced by b-AP15 when compared with bortezomib (Fig. 1E, blue boxes). Both b-AP15 and bortezomib increased the expression of genes associated with oxidative stress; however, the induction of *HMOX1* and *PHOX/p67* expression was ~2.5-fold stronger in b-AP15- compared with bortezomib-exposed cells (Fig. 1E, F; green boxes). The expression of immediate early response genes such as *JUN*, *GADD34*, *GADD45A*, and *GADD45B* were also more strongly induced by b-AP15 compared with bortezomib (Fig. 1E, F). Finally, similar increases in the expression of genes associated with ER stress were observed after treatment with either drug (Fig. 1E, F; red boxes). We conclude that the two drugs induce similar cellular responses; however, b-AP15 elicited a stronger induction of chaperone genes, genes associated with oxidative stress, and immediate early response genes compared with bortezomib.

Inhibition of proteasome function precedes apoptosis after exposure to the DUB inhibitor b-AP15

Cytotoxicity of b-AP15 to tumor cells is associated with induction of apoptosis (10). Analysis of the kinetics of apoptosis induction showed that b-AP15 elicited a more rapid induction of caspase-cleavage of the endogenous cellular substrate keratin-18 (K18) compared with bortezomib (Fig. 2A), with high levels of cleaved K18 appearing 16 h post drug treatment. We previously reported that b-AP15 induces apoptosis in tumor cells genetically defective in *BAX* and in cells overexpressing *Bcl-2* (10). *Bax* and *Bak* are essential for induction of cytochrome c release from mitochondria during apoptosis (62). To extend our previous findings, we used siRNA to down-

regulate the expression of *Bak* in HCT116 colon carcinoma cells. As shown in Figure 2B, down-regulation of *Bak* did not affect apoptosis induction by b-AP15, but inhibited apoptosis induced by bortezomib. The conformation of *Bak* is altered during apoptosis, resulting in increased accessibility of antibodies binding to the N-terminal domain (17). Treatment of HCT116 cells with b-AP15 resulted in increased antibody binding to *Bak*, indicating conformational activation (Fig. 2C). This activation does not, however, appear to be required for b-AP15-induced apoptosis, presumably due to the activation of parallel apoptotic signaling pathways.

We next examined the effects of b-AP15 on *in vivo* proteasome function using a reporter cell line, MelJuSo, expressing the proteasome targeted fusion substrate Ub^{G76V}-yellow fluorescent protein (YFP) (40). This substrate is intrinsically unstable due to constitutive degradation by the proteasome; thus, impairment of the UPS leads to an increased fluorescent signal. At doses below the IC₅₀ (0.25 μ M), a small number of cell nuclei displayed YFP positivity after 1–3 h of b-AP15 drug exposure (Fig. 2D; Supplementary Movie S1 and S2; Supplementary Data are available online at www.liebertpub.com/ars). During the observation period, only these positive cells subsequently rounded up and died. The link between proteasome inhibition and cell death was confirmed using tetramethylrhodamine ethyl ester (TMRE; a fluorescent probe used to monitor the membrane potential of mitochondria), which showed that YFP accumulation preceded loss of mitochondrial membrane potential (Fig. 2E).

b-AP15 induces ER stress

The up-regulation of genes involved in the ER stress response by b-AP15 (Fig. 1D–F) is consistent with previous reports showing induction of ER stress by bortezomib (15,44). As shown in Figure 3A, b-AP15 induced the expression of the ER chaperone Grp78 (BiP) in HCT116 cells. The level of induction was similar to that observed with bortezomib, but weaker than the response to the ER stress-inducer thapsigargin, an inhibitor of sarcoendoplasmic Ca²⁺ ATPase. b-AP15 induced phosphorylation of α -subunit of eukaryotic initiation factor 2 (eIF2- α) already at 6 h post-drug treatment (Fig. 3A). Phosphorylation of eIF2- α is an integral part of the unfolded protein response, by decreasing the load of

FIG. 2. b-AP15-induced apoptotic signaling is distinct from that of bortezomib and apoptosis is preceded by proteasome inhibition. (A) Induction of caspase-cleavage activity by b-AP15 and bortezomib. (Upper panel) HCT116 cells were treated with 1 μ M b-AP15 or 100 nM bortezomib for the times indicated, and cck18 was determined in the cells and medium by the M30 CytoDeath ELISA (Peviva AB). (Lower panel) Dose response to b-AP15 and bortezomib; cck18 was determined at 24 h by ELISA. The pan-caspase inhibitor z-VAD-fmk totally inhibits increases of the signal (see Fig. 3C); (B) HCT116 cells were transfected with an siRNA to BAK or a scrambled control siRNA and incubated for 48 h. Cells were treated with either drug or DMSO solvent and processed for western blotting (top) or M30 CytoDeath ELISA (bottom). Note the insensitivity to b-AP15-induced apoptosis of cells where *Bak* expression has been knocked down. (C) Activation of the conformation of *Bak* by b-AP15. Cells were treated for 6 h, fixed, stained with an antibody to the *Bak* N-terminus, and analyzed by flow cytometry. Gray shaded area: vehicle-treated cells; non-shaded area: b-AP15-treated cells. (D) Analysis of accumulation of a proteasome-targeted fusion protein (Ub^{G76V}-YFP) in MelJuSo cells after exposure to a low dose (0.25 μ M) b-AP15. Note the appearance of YFP in the nuclei of the MelJuSo cells at 1–3 h. Also note that all YFP-positive cells die during the period of observation, whereas cells not becoming YFP positive are unaffected. (E) Analysis of mitochondrial membrane potential ($\Delta\Psi$) and accumulation of Ub^{G76V}-YFP in MelJuSo cells after treatment with 1 μ M b-AP15. $\Delta\Psi$ was determined by staining with TMRE. Cells were analyzed by flow cytometry. Note that YFP positivity precedes the loss of $\Delta\Psi$ (staining with TMRE), showing that proteasome blocking occurs before commitment to apoptosis. cck18, caspase-cleaved keratin 18; z-VAD-fmk, z-Val-Ala-Asp-fluoromethylketone; ELISA, enzyme-linked immunosorbent assay; BAK, BCL2-antagonist/killer; DMSO, dimethyl sulfoxide; YFP, yellow fluorescent protein; TMRE, tetramethylrhodamine ethyl ester.

misfolded proteins in the ER (22), and decreasing translation of cyclin D1 (20). In accordance with previous findings (44), bortezomib failed to induce phosphorylation of eIF2- α . This is due to inhibition of PKR-like ER kinase (PERK) by bortezomib and leads to a lack of translational suppression, and was suggested to be important for the cytotoxic activity of bortezomib (44). The difference between b-AP15 and bortezomib to induce eIF2- α phosphorylation was verified in three additional colon cancer cell lines, whereas a fourth cell line (OXCO-1) displayed some induction by bortezomib (Fig. 3B). The underlying mechanism(s) of the variations in the response of

the cell lines to b-AP15 and bortezomib was not examined further. We conclude that the rapid apoptotic response of HCT116 cells to b-AP15 occurs despite the induction of phosphorylation of eIF2- α .

The ER-associated pro-caspase-4 is the human homolog of mouse caspase-12 (43), and is cleaved after acute ER stress (26,44). Caspase-4 cleavage was detected in a time-dependent manner after bortezomib and b-AP15 treatment (Fig. 3A). To investigate the role of caspase-4 in induction of apoptosis, HCT116 cells were exposed to the caspase-4 inhibitor Z-Leu-Glu-Val-Asp-FMK or transfected with caspase-4 siRNA

FIG. 3. b-AP15 induces endoplasmic reticulum stress. (A) HCT116 cells were exposed to 10 μ M thapsigargin (an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase), 100 nM bortezomib (BZ), or 1 μ M b-AP15 and processed for western blotting. Note the similar induction of Grp78 by all treatments but the differences in induction of eIF2- α phosphorylation. (B) Induction of eIF2- α phosphorylation in CC20, OX-CO-1, CCO-7, and SW-620 colon carcinoma cells. Cells were treated for 6 h as indicated and processed for western blotting. (C, D) b-AP15-induced apoptosis is inhibited by the caspase-4 inhibitor z-LEVD and by caspase-4 siRNA. Transfection with siRNA was performed 48 h before addition of drugs, the caspase-4 inhibitor z-LEVD (20 μ M) or the pan-caspase inhibitor z-VAD (50 μ M) were added at the same time as 100 nM bortezomib or 1 μ M b-AP15. Caspase-cleaved K18 was measured by ELISA at 24 h after drug addition; downregulation of caspase-4 was verified by western blotting. Grp78, glucose-regulated protein 78; eIF2- α , eukaryotic initiation factor 2 alpha; z-LEVD, Z-Leu-Glu-Val-Asp-FMK.

and exposed to either bortezomib or b-AP15. Quantification of the levels of caspase-cleaved K18 showed that inhibition of, or depletion of, caspase-4 reduced both bortezomib- and b-AP15-induced apoptosis (Fig. 3C, D). Taken together, our data suggest that the ER stress induced by b-AP15 contributes to its apoptotic potential.

Role of ROS in b-AP15-induced apoptosis

ROS production has been reported to be a critical mediator of bortezomib-induced apoptosis (15,36,49). The strong induction of *HMOX1* (Fig. 1E), a target gene of redox-regulated nuclear factor-erythroid-2-related factor 2 (Nrf-2), by b-AP15 is consistent with induction of oxidative stress. Induction of *HMOX1* gene expression by b-AP15 in HCT116 cells was confirmed at the protein level by western blotting (Fig. 4A). In MelJuSo Ub^{G76V}-YFP cells, Hmx-1 expression was not further increased using higher concentrations (250 nM) of bortezomib (Fig. 4B). The difference in induction of Hmx-1 by bortezomib and b-AP15 was, therefore, not due to the use of insufficient concentrations of bortezomib.

Experiments using the fluorescent ROS probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) confirmed that b-AP15 induces higher levels of ROS compared with bortezomib (Fig. 5A). To investigate whether ROS was involved in

b-AP15-induced apoptosis, we treated HCT116 cells with the water-soluble vitamin E derivative and antioxidant Trolox. Trolox inhibited apoptosis by ~50% (Fig. 5B). In these experiments, cells were pulsed with b-AP15 for 1 h, after which the drug was removed and fresh media containing Trolox were added. In contrast, no effect on apoptosis was observed when Trolox was present only during the time of drug incubation (Fig. 5B). NAC also inhibited apoptosis induction when added after removal of b-AP15 (Fig. 5C). The inhibitory effects of the ROS scavengers Trolox and NAC on apoptosis are, therefore, not due to a direct chemical reaction with b-AP15 as described for other UPS inhibitors (19), but rather to inhibition of ROS generation downstream from b-AP15-mediated proteasome inhibition. However, when we analyzed the effect of Trolox on rescuing cells from ROS-induced death, no reduction in cytotoxicity was observed (Supplementary Fig. S1). This was in contrast to bortezomib, where Trolox partially inhibited drug-induced cell death (Supplementary Fig. S1). These findings show that apoptosis induction by b-AP15 involves oxidative stress, but that other mechanisms will eventually cause cell death when oxidative stress is alleviated.

Sustained oxidative stress is known to impair the functionality of the UPS (9,13). The activity of the proteasome appears to be more sensitive to oxidative stress than the DUBs

FIG. 4. Induction of Hmox-1 [heme oxygenase (decycling) 1] protein expression by b-AP15 and bortezomib. (A) HCT116 cells were exposed to 100 nM bortezomib or 1 μ M b-AP15 for 18 h and processed for western blotting using antibodies to Hmox-1 or β -actin. (B) MelJuSo Ub^{G76V}-YFP cells were exposed to the indicated concentrations of bortezomib or b-AP15 for 18 h and processed for western blotting using antibodies to ubiquitin, Hmox-1, or β -actin. Note that the use of increasing concentration of bortezomib does not result in further induction of Hmox-1.

(66). The finding of strong induction of oxidative stress by b-AP15, therefore, raised the possibility that alterations in cellular redox status contribute to inhibition of proteasome function. To examine this question, we exposed MelJuSo Ub^{G76V}-YFP reporter cells to bortezomib or b-AP15 in the presence and absence of Trolox as an ROS scavenger (Fig. 5D). The presence of Trolox during the drug treatment had no effect on the accumulation of the Ub^{G76V}-YFP reporter, con-

firmed that both drugs inhibit proteasome activity independently of ROS levels (Fig. 5D). We also examined whether proteasome function was affected by rotenone, an agent that interferes with mitochondrial electron transport and induces ROS. No effect on Ub^{G76V}-YFP accumulation in cells was observed (Fig. 5D).

To investigate the biological effects of ROS induced by proteasome inhibition, we studied the effect of scavengers on gene expression after treatment with b-AP15. Pretreatment with Trolox resulted in a lower level of expression of a number of stress-induced genes after proteasome inhibition (Fig. 5E). As expected, the expression of the oxidative stress-induced genes *HMOX1* and *PHOX/p67* was reduced by cotreatment with Trolox. The induction of genes regulated by HSF-1 was also reduced; *HSPA6* by approximately fivefold, *HSPA1B* and *DNAJB1* (Hsp40) by approximately twofold. The reduction in chaperone expression after Trolox cotreatment may be due to alleviation of ROS-induced protein oxidation (2). The relationship between chaperone and ROS expression would fit with previous reports showing ROS induction of Hsp70 expression (39). Interestingly, b-AP15 induction of ER stress-regulated genes was not significantly affected by Trolox (Fig. 5E), suggesting that ER stress was independent from oxidative stress.

We finally examined the ability of a number of b-AP15 analogues to induce Hmox-1 and to induce the formation of high-molecular-weight poly-ubiquitin conjugates in HCT116 cells. We found that compound NSC687849 generated accumulation of poly-ubiquitin conjugates to a similar extent as b-AP15, whereas three other tested analogues did not show activity at 1 μ M (Fig. 6). Interestingly, Hmox-1 levels were increased by b-AP15 and NSC687849, but not by the other compounds, showing an association between proteasomal blocking and induction of oxidative stress.

Induction of stress-activated protein kinases

We examined the gene expression response to b-AP15 in greater detail using Gene Set Enrichment Analysis (GSEA) of microarray data (54). Consistent with the qPCR results shown earlier, we found strong enrichment of genes containing HSF-1-regulated promoters (Fig. 7A). Interestingly, the GSEA also showed a strong enrichment of genes regulated by the activating protein-1 (AP-1) transcription factor (Fig. 7B). Both b-AP15 and bortezomib induced induction of phosphorylation of Jun-N-terminal kinase 1/2 (JNK) in HCT116 cells (Fig. 7C). Phosphorylation of JNK could be detected at 1–2 h after exposure to b-AP15, time points when bortezomib did not induce JNK phosphorylation (Fig. 7C, D). Increasing the concentration of bortezomib to 1 μ M did not result in detectable induction of JNK phosphorylation at 2 h (Fig. 7D). At 6 and 8 h, total c-Jun protein levels were increased in both b-AP15- and bortezomib-treated cells (Fig. 7E), as expected from positive autoregulation (3). Increases in the phosphorylation of p38-MAPK were also observed after exposure to b-AP15 and to bortezomib (Supplementary Fig. S2A). Experiments using the JNK inhibitor SP600125 and the p38-MAPK inhibitor SB203580 showed that, as expected, stimulation of phosphorylation of c-Jun at Ser-73 was due to JNK activity (Fig. 7F). NAC was found to reduce JNK activation by b-AP15 in experiments in which the scavenger was added after removal of b-AP15 (Fig. 7G), suggesting that JNK activation was mediated by ROS. We finally examined whether activation of

JNK and p38-MAPK was required for apoptosis induction by b-AP15 and bortezomib. Pharmacological inhibition of JNK was found to inhibit apoptosis induced by b-AP15 (Fig. 7H), whereas inhibition of p38-MAPK did not affect apoptosis (Supplementary Fig. S2B).

Discussion

The present study establishes that proteasome inhibition by the USP14/UCHL5 DUB inhibitor b-AP15 induces a distinct cellular response compared with that of the 20S inhibitor bortezomib. The two agents elicit similar, but yet distinct, patterns of cytotoxicity on the NCI₆₀ cancer cell line panel. Further, although both proteasome inhibitors induce apoptosis, the kinetics of b-AP15-induced caspase activation was significantly more rapid (>6 h time difference between treatments). The difference in the timing of apoptosis induction could not be explained by different kinetics of proteasome inhibition (Fig. 5D). Different apoptosis signaling pathways also appear to be induced in response to the two types of proteasome inhibitors, as b-AP15 does not require Bak or Bax for activation of caspase-cleavage activity [Fig. 2B and (10)]. The fact that the response to two agents both of which block the proteasome and that both increase the levels of proteins such as p53, p21, and p27 (10) differs raises questions with regard to the relative importance of stabilization of cellular regulatory proteins for triggering apoptosis. We hypothesize that the distinct cellular response to b-AP15 is related to the strong proteotoxic response induced by this drug, as evidenced by the >1000-fold induction of the HSPA6 chaperone gene by b-AP15. The mechanism underlying this elevated proteotoxicity is not clear but may be due to the accumulation of misfolded proteins with long polyubiquitin chains occurring as a consequence of inhibition of the USP14/UCHL5 DUBs (10). It is possible that misfolded proteins bearing longer ubiquitin chains are processed differently by the cell, resulting in stronger proteotoxic stress. Another possibility is that inhibition of other, non-proteasome-associated, activities of USP14 by b-AP15 will contribute to apoptosis induction [see (41)].

Oxidative stress has been associated with apoptosis induced by proteasome inhibitors (15,36,49). While our results do not contribute to the understanding of why proteasome inhibitors induce oxidative stress, they clearly establish a role of ROS in the induction of apoptosis by b-AP15. Whether ROS is involved in apoptotic signaling in cells exposed to proteasome inhibitors is controversial, as a number of ROS scavengers such as NAC and vitamin C form complexes with bortezomib, piperlongumine, and other UPS inhibitors

(14,19,37,68). Here, we found that b-AP15 induced an ~40-fold induction of *HMOX1* expression, a gene known to be regulated by Nrf-2 and induced in response to oxidative stress (42). The amplitude of *HMOX1* induction by b-AP15 was significantly higher compared with bortezomib and was concomitant with ROS induction measured by DCFH-DA fluorescence. We found that apoptosis induction by b-AP15 was decreased by Trolox, whereas proteasome blocking was not impaired. By sequential exposure of cells to b-AP15 and scavengers (Trolox or NAC), we ascertained that inhibition of apoptosis by scavengers was not due to an interaction with the drug. Our findings, therefore, establish that ROS is a key mediator of apoptosis induction by b-AP15.

Oxidative stress is known to activate the stress-activated kinases p38-MAPK and JNK through ASK1 (16,29), and these pathways are known to be induced by proteasome inhibitors (25,65). Further, yeast strains carrying mutations in 26S proteasome subunits overexpress Pap1, the *Schizosaccharomyces pombe* homolog of the human AP-1 transcription factor (48). We indeed found activation of both p38-MAPK and JNK by b-AP15. Similar to previous reports on bortezomib (25,65), a pharmacological inhibitor of JNK impaired b-AP15-induced apoptosis, whereas inhibition of p38-MAPK did not. As expected, phosphorylation of JNK was impaired by treatment with NAC. The relatively rapid induction of JNK implied that early induction of apoptosis is associated with activation of this pathway. These results establish JNK induction as a downstream mediator of b-AP15-induced oxidative stress.

Apoptosis by bortezomib has been attributed to the induction of ER stress (11,15,44,58). b-AP15 was also found to induce ER stress as evidenced by increased expression of *CHOP* (CCAAT/enhancer-binding protein (C/EBP) homologous protein; GADD153), *HERPUD1*, *GRP78*, and *XBP1* expression. *CHOP* is known to be induced by ER stress and by oxidative stress (67). We found that the expression of ER-stress responsive genes after b-AP15 was not affected by Trolox, showing that oxidative stress was not involved in mediating ER stress. A major difference in the ER stress response to b-AP15 and bortezomib was the induction of phosphorylation of the eIF2- α by b-AP15. eIF2- α is usually phosphorylated during ER stress by PERK, leading to attenuation of protein synthesis (31). Such translational attenuation will reduce protein synthesis and, thus, reduce accumulation of misfolded proteins in the ER (22), thereby alleviating ER stress and promoting cell survival. Bortezomib does not induce eIF2- α phosphorylation, due to inhibition of PERK, and the lack of attenuation of protein synthesis during ER stress has been

FIG. 5. b-AP15-induced apoptosis is inhibited by ROS scavengers. (A) Induction ROS by bortezomib or b-AP15 in HCT116 cells. Cells were stained with DCFH-DA after 6 h of treatment and analyzed by flow cytometry. **(B)** b-AP15-induced apoptosis is inhibited by the ROS scavenger Trolox. Cells were treated with 1 μ M b-AP15 for 1 h, and Trolox (5 mM) was added either during (0–1 h) or after (1–24 h) drug treatment. Caspase-cleaved K18 was determined by ELISA. **(C)** b-AP15-induced apoptosis is inhibited by the ROS scavenger NAC. Cells were treated with 1 μ M b-AP15 for 1 h, and 5 mM NAC was added after drug treatment. Caspase-cleaved K18 was determined by ELISA after 24 h. **(D)** Accumulation of Ub^{G76V}-YFP in MelJuSo cells is not affected by Trolox. Cells were treated with 100 nM bortezomib, 1 μ M b-AP15, or 50 μ M rotenone in the presence or absence of 5 mM Trolox. Note the similar kinetics of induction of Ub^{G76V}-YFP in cells exposed to bortezomib or b-AP15, and note the lack of effect of Trolox on YFP positivity. Rotenone induces strong induction of ROS in HCT116 cells (30) but does not induce YFP positivity. **(E)** Effects of Trolox on the expression of stress-associated genes. The expression of the 84 gene-set used in Figure 1 was examined in cells treated with b-AP15 in the presence or absence of 5 mM Trolox. Note the reduction of expression of genes regulated by HSF-1 and genes associated with oxidative stress in cells cotreated to Trolox, whereas genes associated with ER stress are not affected. ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein diacetate; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; NAC, N-acetyl cysteine.

We previously showed that b-AP15 induces lysosomal membrane permeabilization and apoptosis dependent on the lysosomal protease cathepsin-D (4). Interestingly, several apoptotic agents have been described to trigger lysosomal membrane permeabilization and induce cell death by generating oxidative stress (12,47,53,59). Although the precise mechanism is unclear, increased production of ROS may lead to destabilization

FIG. 6. Association between induction of Hmx-1 and accumulation of high-molecular-weight poly-ubiquitin. Cells were treated with the indicated compounds at a concentration of 1 μ M. Cells were processed for western blotting using the indicated antibodies. Compounds were gifts from the National Cancer Institute; shown are cancer chemotherapy National Service Center numbers. Further information on these compounds is available using the search function at <http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp>

of the lysosomal membrane *via* massive peroxidation of membrane lipids. The present observations of high levels of ROS induction by b-AP15 would be consistent with these results.

We conclude that the strong proapoptotic potential of the DUB inhibitor b-AP15 is associated with induction of oxida-

tive stress. This is common territory in the field of cancer therapy, as, in fact, most of our clinically used cancer drugs, as well as ionizing radiation, induce oxidative stress (61). Malignant transformation is known to be associated with enhanced cellular stress, including both oxidative and proteotoxic stress, and the strong oxidative stress associated with b-AP15 may be poorly tolerated by cancer cells, providing a rationale for therapy of a wide variety of malignancies (38,57).

Materials and Methods

Materials

b-AP15 was obtained from Oncotargeting AB, bortezomib from ApoEx, SP600125 from Sigma-Aldrich, and SB203580 from Cell Signaling. Compounds were dissolved in dimethyl sulfoxide. Control cultures received the same final concentration of solvent as treated ones.

Cell culture

HCT116 colon carcinoma cells were maintained in McCoy's 5A modified medium/10% fetal calf serum. The proteasome reporter cell line MelJuSo Ub-YFP was generated as described (40) and was cultured in Dulbecco's modified Eagle's medium/10% fetal calf serum. All cells were maintained at 37°C in 5% CO₂ and subcultured by trypsinization.

Assessment of apoptosis

HCT116 cells were seeded in 96-well microtiter plates at 10,000 cells per well and incubated overnight. Drugs were then added and cells were incubated further. At the end of the incubation period, NP40 was added to the tissue culture medium to 0.1% and 25 μ l of the content of each well was assayed using the M30-CytoDeath[®] enzyme-linked immunosorbent assay (ELISA) (Peviva AB) (28). This ELISA is based on a specific antibody against a neopeptide of K18 that is generated by the action of caspase-3, -7- and -9 activated in response to apoptosis (34).

Measurement of Bak-associated immunofluorescence

Cells were fixed in paraformaldehyde (0.25%, 5 min) before incubation for 1 h with an antibody recognizing an N-terminal

FIG. 7. b-AP15-induced apoptosis is dependent on activation of AP-1. (A, B) Gene set enrichment analysis based on b-AP15 treatment of HCT116 cells (1 μ M, 6 h exposure). The genes have been rank ordered according to their relative expression after b-AP15 exposure compared with vehicle control. The enrichment profiles (green areas) show the enrichment of genes (represented by black vertical lines) containing binding sites for HSF-1 and AP-1, respectively. Nominal *p*-values < 0.001; NES = normalized enrichment score (54). (C) Induction of phosphorylation of JNK1/2 by b-AP15 (1 μ M) or bortezomib (100 nM). HCT116 cells were exposed to the drugs for the indicated time in hours. Note the detection of JNK phosphorylation at 1 and 2 h of exposure to b-AP15, but not to bortezomib; (D) Induction of phosphorylation of JNK1/2 b-AP15, but not by bortezomib, at 2 h of drug exposure. HCT116 cells were exposed to the indicated concentration of drugs. (E) Induction of phosphorylation of c-Jun by b-AP15 (1 μ M) or bortezomib (100 nM). HCT116 cells were exposed to the drugs for the indicated time in hours. (F) Induction of c-Jun phosphorylation by b-AP15 (1 μ M) or bortezomib (100 nM) is inhibited by the JNK inhibitor SP600125 (10 μ M), but not by the p38-MAPK inhibitor SB203580 (10 μ M). HCT116 cells were treated for 6 h. Note that c-Jun phosphorylation is inhibited by SP600125, whereas JNK phosphorylation is only marginally inhibited. (G) NAC (5 mM) inhibits b-AP15-induced phosphorylation of JNK. HCT116 cells were exposed to b-AP15 (1 μ M), followed by washout and addition of NAC. Numbers below the blots represent the p-JNK/JNK ratios. (H) The JNK inhibitor SP600125 inhibits b-AP15-induced caspase-cleavage of K18. HCT116 cells were treated with 1 μ M b-AP15 or 100 nM bortezomib for 24 h in the presence or absence of 10 μ M SP600125. Caspase-cleaved K18 was determined by ELISA. AP-1, activating protein-1; JNK, Jun N-terminal kinase.

epitope of Bak that is accessible only after conformational activation (AM03; Oncogene Research Products) (17). The primary antibody was diluted in phosphate buffered saline (PBS) containing digitonin (100 $\mu\text{g}/\text{ml}$). Cells were then incubated for 1 h with an Allophycocyanin-conjugated secondary antibody in PBS (550826; BD Biosciences Pharmingen), and fluorescence was measured by flow cytometry.

Measurement of DCFH-DA fluorescence

HCT116 cells were treated with b-AP15, bortezomib, or solvent; stained using DCFH-DA; and subsequently analyzed

using an FL1-H wavelength channel of a fluorescence-activated cell sorting (FACS) Calibur flow cytometer.

siRNA knock-down

HCT116 cells were transfected with siRNA (Qiagen) against human BAK, BAX, or a scrambled control sequence. Two days later, the transfected cells were trypsinized and seeded in 96-well plates. The day after seeding, drugs were added to the cells and drug treatment was allowed to continue for 24 h, after which analysis of apoptosis using the M30-Apoptosense ELISA was conducted.

Western blot analysis

For ubiquitin analysis, cell extract proteins were resolved by Tris-Acetate polyacrylamide gel electrophoresis gels (Invitrogen) and transferred onto a polyvinylidene difluoride membrane for western blotting. Antibodies were obtained from the following sources: c-JUN (BD Transduction Laboratories), p-cJUN (Ser73), eIF2- α , p-eIF2- α -JNK (T183/Y185) (Cell Signaling), JNK (Santa Cruz), HMOX1, GRP78 (BD Transduction Laboratories), ubiquitin (Chemicon), and β -actin (Sigma-Aldrich).

Live-cell analysis of UPS activity

MelJuSo Ub^{G76V}-YFP cells (40) were plated in black optically clear bottom ViewPlates (PerkinElmer) overnight and then treated with b-AP15 or bortezomib. Treatment with compounds that block the UPS leads to accumulation of YFP in these cells, and the generated fluorescence was continuously detected in an IncuCyte FLR instrument (Essen BioScience Inc.).

Transcriptional profiling and GSEA

We examined the pattern of transcripts for 84 genes that were reported to respond to stress. The identity of these genes is given in Supplementary Table S1. Cells were exposed to b-AP15 or bortezomib for 6 h in the presence or absence of Trolox; cDNA was prepared from 1 μ g of RNA using the RT² First-Strand Kit (SA Biosciences). All primers used for qPCR were purchased from SA Biosciences Corp. The array analysis was carried out following the manufacturer's protocol with SYBR Green PCR (ROX) Master Mix (SA Biosciences Corp.). Arrays were assayed for 1 cycle of 10 min at 95°C, then 40 cycles of (15 s at 95°C, followed by 1 min at 60°C) using an ABI7500 system. All conditions were performed in triplicate and normalized to five housekeeping gene mRNA levels. To analyze the PCR-array data, SA Biosciences Web-portal software was used: <http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>

The GSEA software and method for microarray result exploration has been described elsewhere (54). Briefly, the pre-ranked gene list from b-AP15 cells *versus* untreated control (10) was compared with a priori defined and curated gene sets from the MSigDB database (*i.e.*, V\$AP1_01 with systematic name M10220 and V\$HSF_Q6 with systematic name M16482), using standard GSEA settings. The purpose of GSEA is to find out whether the a priori defined gene sets are significantly enriched toward the upper or lower end of the pre-ranked list. The *p*-value refers to the nominal *p*-values after 1000 permutations.

Measurement of mitochondrial membrane potential

Loss of mitochondrial membrane potential was assessed by incubating cells with 25 nM TMRE (Invitrogen) for 30 min. Changes in mitochondrial membrane potential ($\Delta\Psi$) were monitored in an FACS Calibur flow cytometer.

Statistical analysis

Statistical analysis (Student's *t*-test, Pearson correlation coefficient, or Spearman's rank correlation coefficient, as indicated) was performed using Prism software for Apple

computers. Statistical significance in *t*-test was plotted as follows: * (*p* < 0.05), ** (*p* < 0.01), and *** (*p* < 0.001).

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M.F., R.L., and S.L. own shares in Vivolux AB, which develops cancer drugs based on proteasomal inhibition. No competing financial interests exist for the other authors.

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Abbreviations Used

19SRP = 19S regulatory particle
 20SCP = 20S core particle
 AP-1 = activating protein-1
 BAK = BCL2-antagonist/killer
 BAX = BCL2-associated X protein
 Bcl-2 = B-cell CLL/lymphoma 2
 ccK18 = caspase-cleaved keratin 18
 CHOP = CCAAT/enhancer binding protein (C/EBP) homologous protein
 DCFH-DA = 2',7'-dichlorofluorescein diacetate
 DMSO = dimethyl sulfoxide
 DNAJB1 = DnaJ (Hsp40) homolog, subfamily B, member 1
 DUB = deubiquitinase 19SRP
 eIF2- α = eukaryotic initiation factor 2 alpha
 ELISA = enzyme-linked immunosorbent assay
 ER = endoplasmic reticulum
 FACS = fluorescence-activated cell sorting
 GADD = growth arrest and DNA damage
 Grp78 = glucose regulated protein 78
 GSEA = Gene Set Enrichment Analysis
 HERPUD1 = homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1
 HMOX1 = heme oxygenase 1

HSF-1 = heat shock factor protein 1
 HSPA6, HSPA1B = heat shock protein encoding genes
 IC₅₀ = 50% inhibitory concentration
 JNK = Jun N-terminal kinase
 JUN = jun proto-oncogene
 K18 = keratin-18
 NAC = N-acetyl-cysteine
 NCI = National Cancer Institute
 NFkB = nuclear factor kappa B
 Nrf-2 = nuclear factor-erythroid-2-related factor 2
 PBS = phosphate-buffered saline
 PERK = PKR-like ER kinase
 PHOX/p67 = neutrophil cytosolic factor 1, NCF2
 qPCR = quantitative polymerase chain reaction
 ROS = reactive oxygen species
 TMRE = tetramethylrhodamine ethyl ester
 Trolox = 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
 UCHL5 = ubiquitin carboxyl-terminal hydrolase L5
 UPS = ubiquitin proteasome system
 USP14 = ubiquitin specific peptidase 14
 XBP1 = X-box binding protein 1
 YFP = yellow fluorescent protein
 z-LEVD = Z-Leu-Glu-Val-Asp-FMK