Role of CAAT/Enhancer Binding Protein Homologous Protein in Panobinostat-Mediated Potentiation of Bortezomib-Induced Lethal Endoplasmic Reticulum Stress in Mantle Cell Lymphoma Cells

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Abstract

Purpose: Bortezomib induces unfolded protein response (UPR) and endoplasmic reticulum stress, as well as exhibits clinical activity in patients with relapsed and refractory mantle cell lymphoma (MCL). Here, we determined the molecular basis of the improved in vitro and in vivo activity of the combination of the pan-histone deacetylase inhibitor panobinostat and bortezomib against human, cultured, and primary MCL cells.

Experimental Design: Immunoblot analyses, reverse transcription-PCR, and immunofluorescent and electron microscopy were used to determine the effects of panobinostat on bortezomib-induced aggresome formation and endoplasmic reticulum stress in MCL cells.

Results: Treatment with panobinostat induced heat shock protein 90 acetylation; depleted the levels of heat shock protein 90 client proteins, cyclin-dependent kinase 4, c-RAF, and AKT; and abrogated bortezomib-induced aggresome formation in MCL cells. Panobinostat also induced lethal UPR, associated with induction of CAAT/enhancer binding protein homologous protein (CHOP). Conversely, knockdown of CHOP attenuated panobinostat-induced cell death of MCL cells. Compared with each agent alone, cotreatment with panobinostat increased bortezomib-induced expression of CHOP and NOXA, as well as increased bortezomib-induced UPR and apoptosis of cultured and primary MCL cells. Cotreatment with panobinostat also increased bortezomib-mediated in vivo tumor growth inhibition and improved survival of mice bearing human Z138C MCL cell xenograft.

Conclusion: These findings suggest that increased UPR and induction of CHOP are involved in enhanced anti-MCL activity of the combination of panobinostat and bortezomib.

Mantle cell lymphoma (MCL) is an aggressive, well-defined subset of B-cell non–Hodgkin’s lymphoma, which accounts for nearly 6% of all lymphomas (1, 2). It is characterized by deregulated expression of cyclin D1, due to the CCND1-IgH translocation, resulting from the chromosomal translocation t(11;14; q13;q32) (refs. 2, 3). In addition, MCL is often associated with expression of a truncated cyclin D1 variant, enhanced activity of NF-κB and AP1, genomic amplification of cyclin-dependent kinase 4 (CDK4), deletion of the CDK inhibitor p16INK4a, and overexpression of BMI-1, a transcriptional repressor of the p16INK4a locus (2, 3). MCL patients respond initially to chemotherapy and autologous stem cell transplantation with an overall survival of ~3 to 4 years (4). However, after an initial response, a relapse is typical and chemoresistance is common (4). Several recent studies have documented clinical responses and benefit in MCL following treatment with a variety of novel agents. These include the mammalian target of rapamycin (mTOR) kinase inhibitor temsirolimus, proteasome inhibitor bortezomib, and the immunomodulatory agent lenalidomide (4–6). However, none of these agents provide long-term benefit, and patients eventually succumb to the disease (4). These factors clearly indicate the necessity to develop novel combination therapies for the treatment of MCL.

Bortezomib is a clinically effective agent in relapsed and refractory MCL (6). Bortezomib exerts its anti-MCL activity...
through multiple mechanisms (7, 8). These include inhibition of NF-κB, stabilization of p53, generation of reactive oxygen species, induction of the BH3 domain-only protein NOXA, accumulation of misfolded proteins, as well as induction of protracted and lethal endoplasmic reticulum (ER) stress (7–9). Recently, pan-histone deacetylase (HDI) inhibitors (HDI), for example, vorinostat and panobinostat, were also documented to have clinical activity against a variety of hematologic malignancies (10–12). HDI treatment induces cell cycle growth arrest and apoptosis of transformed more than normal cells through multiple mechanisms (13). For example, treatment with panobinostat has been shown to increase reactive oxygen species production, suppress cyclin D1, induce cell cycle–dependent kinase inhibitors p21 and p27, as well as induce the levels of the proapoptotic proteins, e.g., BAX, BAK, and BIM, in leukemia and other transformed cell types (13, 14). Further, in some transformed cells, HDI treatment is known to decrease the levels of antiapoptotic proteins, e.g., Bcl-xL, MCL-1, XIAP, survivin, and AKT, thereby lowering the threshold for apoptosis (13, 14). In previous reports, treatment with panobinostat was shown to inhibit HDAC6, induce heat shock protein 90 (hsp90) acetylation, and disrupt chaperone association of hsp90 with its client proteins, including AKT, CDK4, and c-Raf, thereby promoting misfolding, polyubiquitylation, and proteasomal degradation of the hsp90 client proteins (15–17). By inhibiting HDAC6, HDI treatment also abrogates formation of aggresome, which normally serves to sequester and protect against misfolded polyubiquitylated proteins (18). Consistent with this, HDI treatment has been shown to induce unfolded protein response (UPR) and ER stress (19). Disruption of ER homeostasis and the resulting proteotoxicity has been recognized as a novel mechanism for inhibiting tumor cell proliferation and survival, especially in B-cell malignancies (20, 21). Typically, UPR is an adaptive response to misfolded proteins in the ER, which activates the ER membrane-bound mediators of ER stress response, inositol requiring enzyme (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6α (ATF6α; refs. 22, 23). Accumulation of misfolded proteins causes the dissociation of the ER chaperone glucose-regulated protein 78 (GRP78) from the mediators of ER stress response, leading to their activation, with ensuing upregulation of gene expression involved in either the alleviation of ER stress or cell death (22–24). UPR induces PERK-mediated phosphorylation of eukaryotic initiation factor 2α (elF2α), which blocks cap-dependent protein translation but allows preferential translation of ATF4, a transcription factor that upregulates chaperone proteins required to restore ER function and induces the prodeath transcriptional regulator CAAT/enhancer binding protein homologous protein (CHOP; refs. 22–24). CHOP is composed of an NH2-terminal transactivating domain and a COOH-terminal basic-leucine zipper domain. It functions by heterodimerizing with the CAAT/enhancer binding protein family of proteins and inhibiting their DNA binding or by binding to an alternate unique site to activate target genes (22–24). ATF6 activation leads to upregulation of genes that have an ER stress response element in their promoters such as GRP78, GRP94, CHOP, and X-box binding protein 1 (XBP1; refs. 22–24). Activated IRE1 catalyses the splicing of XBP1 to generate a frameshift splice variant of XBP1 called XBP1s, which induces GRP78 and other proteins involved in restoring normal ER function (22–24). However, persistent proteotoxic stress with unresolved UPR and ER stress results in protracted PERK signaling and CHOP-mediated cell death (23–25). Consistent with this, combined treatment with bortezomib and an hsp90 inhibitor, which enhances UPR, has been shown to induce synergistic lethal effects in B-cell malignancies (26). In the present study, we determined the molecular basis of panobinostat-mediated potentiation of bortezomib-induced UPR and ER stress, resulting in lethal effects in cultured and primary MCL cells. Our findings show that, by inducing acetylation and inhibition of hsp90 chaperone function and concomitantly abrogating bortezomib-induced aggresome formation, panobinostat accentuates bortezomib-induced UPR and ER stress in MCL cells. This is associated with increased CHOP expression due to co-treatment with panobinostat and bortezomib, resulting in synergistic in vitro and in vivo cytotoxicity against human MCL cells. Our findings also show that CHOP knockdown inhibits panobinostat-mediated cell death of MCL cells.

Materials and Methods

Reagents and antibodies

Panobinostat was provided by Novartis Pharmaceutical, Inc. Phosho-elF2α, elF2α, cyclin D1, AKT, Bak, and PUMA were purchased from Cell Signaling Technology.
CDK4, HDAC6, GRP78, cyclin D1, c-Myc, Bax, ATF4, and CHOP antibodies were purchased from Santa Cruz Biotechnology, Inc. Monoclonal hsp90 antibodies were obtained from Assay Designs. Antibodies to acetylated lysine, eIF2α, phospho-eIF2α, and Bak antibodies were purchased from Cell Signaling Technology. NOXA antibody was obtained from Abcam; c-Raf antibodies were purchased from BD Transduction Laboratories; and ubiquitin antibodies were obtained from Covance.

MCL cell lines and primary MCL cells

Human MCL cell lines JeKo-1 and Z138C were obtained from the American Type Culture Collection and cultured in RPMI 1640 containing 20% heat-inactivated fetal bovine serum. MO2058 cells were cultured as previously described (26, 27). Granta-519 cells were obtained from DSMZ and cultured in DMEM containing 20% fetal bovine serum. Logarithmically growing cells were exposed to the designated concentrations and exposure interval of the drugs. Following these treatments, cells were pelleted and washed free of the drug(s) before the performance of the studies described below. Primary MCL cells were obtained with informed consent as part of a clinical protocol approved by the institutional review board of the Medical College of Georgia. Bone marrow and/or peripheral blood samples were collected in heparinized tubes, and mononuclear cells were separated using Lymphoprep (Axis-Shield), washed once in complete RPMI, and counted to determine the number of isolated cells before their use in the experiments. Percentage cell death of primary MCL cells following 48 hours of exposure to panobinostat and bortezomib was assessed in triplicates by trypsin blue dye exclusion assay and a hemocytometer. Values are presented as percentage cell death ± SD.

Western blot analyses and immunoprecipitation

Western blot analyses were done using specific antisera or monoclonal antibodies as described previously (14–16). The expression of β-actin was used as a loading control in immunoblot analysis. Data presented are representative of at least three independent experiments. Following drug treatments, cell lysates were incubated with 2 μg of hsp90 antibody, immunoprecipitated as previously described, and probed with anti-acetylated lysine antibody to assess hsp90 acetylation. Immunoprecipitated hsp90 was detected by stripping the blot and immunoblotting with an hsp90 antibody (Assay Designs; ref. 15).

ATP binding assay

MCL cells were incubated with indicated doses of panobinostat for 16 hours and washed free of drugs. ATP-bound proteins were immunoprecipitated from the resulting cell lysates by incubating with ATP-Sepharose (Innova Biosciences) beads for 4 hours. Following four washes, ATP-bound proteins were boiled with SDS loading buffer and resolved on a 10% SDS-PAGE gel. ATP-bound hsp90 was detected by immunoblotting with anti-hsp90 antibody (Assay Designs; ref. 15).

Reverse transcription-PCR

Total RNA was isolated from JeKo-1 cells and primary MCL cells using Trizol reagent (Invitrogen) as per manufacturer’s instructions. Reverse transcription was done with 2 μg RNA using SuperScript RT (Invitrogen). PCR reactions were done using the corresponding specific primers listed below and 2× IQ Supermix (Bio-Rad) reagent. The identity of PCR products was confirmed by sequencing. β-Actin was used as a loading control. The authenticity of all PCR products was confirmed by sequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
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<tr>
<td>XBP1s</td>
<td>Forward primer 5′-TCTGCTGAGTCCGCAGCAG-3′</td>
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<tr>
<td></td>
<td>Reverse primer 5′-GAAAAGGAGGCTTGTGAAGGAAC-3′</td>
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<td>XBP1u</td>
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<td>Reverse primer 5′-TGGTTGCTAGAGAGAGCGAGG-3′</td>
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<tr>
<td></td>
<td>Reverse primer 5′-GGTATATAGTACGCCCCAAGG-3′</td>
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<td></td>
<td>Reverse primer 5′-CCACITTCCTTATTCATCTTG-3′</td>
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<td></td>
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<tr>
<td>ER oxidase 1α</td>
<td>Forward primer 5′-GAATTCTTGAGTGAGAAGGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5′-TCTACAGACAGCCTCTAGG-3′</td>
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Confocal microscopy

MCL cells were incubated with indicated doses of drugs and cytospun onto slides. Then, cells were fixed with 4% paraformaldehyde for 10 minutes, washed, permeabilized with 0.5% Triton-PBS buffer for 5 minutes, and stained with HDAC6 and ubiquitin antibodies as described previously (16). The images were visualized using Carl Zeiss LSM-510 meta confocal microscope with a 63×/1.2 W objective.

Ultrastructural studies

MCL cells were exposed to bortezomib and/or panobinostat for 16 hours. For transmission electron microscopy,
MCL cells were fixed in 2% glutaraldehyde postfixed in 2% osmium tetroxide, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series, and embedded in Epon-Araldite resin. Thin sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Inc.), collected on copper grids, and stained with uranyl acetate and lead citrate. Cells were observed in a JEM 1230 transmission electron microscope (JEOL USA, Inc.) at 80 kV and imaged with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan, Inc.).

**Tubulin deacetylase activity**

Recombinant HDAC6 (Bio Vision) was incubated with equal concentrations of tubulin deacetylase substrate (Biomol International, L.P., PA) and increasing concentrations of panobinostat for 30 minutes in HDAC assay buffer (Biomol International, L.P., PA). The samples were then incubated with Fluor de Lys developer, and fluorescence intensity was measured using a fluorimeter from BioTek Instruments, Inc. Inhibition of HDAC6 activity was expressed as percentage of untreated control values.

**Annexin V-propidium iodide staining**

MCL cell lines were exposed to indicated concentrations of drugs for 48 hours and pelleted. Following washes with PBS, they were stained with Annexin V-FITC (BD Biosciences) and propidium iodide (PI). Apoptotic cells were assessed using a FACSCalibur flow cytometer as described previously (14–16). Synergistic interactions were assessed using the median dose effect of Chou-Talalay. Combination indices for the two drugs were assessed using the median dose effect of Chou-Talalay. Combination indices for the two drugs were obtained using the commercially available software Calcsyn (Biosoft; ref. 26).

**Transfection**

JeKo-1 cells were transiently transfected using Turbofectin 8.0 (Origene) as per the manufacturer’s instructions, with 3 μg plasmid vector pBS/U6 with or without HDAC6 siRNA (15), which had a 21-nucleotide sequence (‘5’-GAATGGATCTGAACCTTGAGA-3’) corresponding to targeted nucleotides 200 to 219 in HDAC6 mRNA (accession no. BC013737).

**Retroviral knockdown of CHOP**

siRNA to knockdown CHOP mRNA was designed using siRNA Target Finder software (Ambion, Inc.). Oligonucleotides were denatured, annealed, and cloned into pSilencer 5.1 H1 Retro vector using the BamHI and HindIII restriction sequences. The sequences of the siRNA duplex used in the study are as follows:

- Sense strand siRNA: CCAGGAAAAGGAAACAGAGtt
- Antisense strand siRNA: CUCUGUUIUCCGUUUC-CUGGtGtt

**Retrovirus production and retroviral infection**

HEK-GP2 (host) cells were cotransfected with vesicular stomatitis virus-G (envelope protein) and pSilencer 5.1 H1 carrying scrambled oligos (control siRNA) or siCHOP oligos using Cal-Phos transfection reagent from Clontech. Two days after transfection, the culture supernatants carrying the retroviral particles were used for infecting JeKo-1 cells.

**In vivo studies with MCL cell xenograft**

Five million Z138C cells were implanted into the flanks of nonobese diabetic/severe combined immunodeficient mice subcutaneously. Mice were divided into four groups (n = 8). Tumors were allowed to grow, and the treatment was commenced when the average tumor volume reached 50 mm³. Control mice received DMSO. Panobinostat was administered at a dose of 10 mg/kg three times a week (days 1, 3, and 5). Bortezomib was administered at a dose of 0.5 mg/kg once a week (day 2). No drugs were administered on days 6 and 7. Mice receiving the combination therapy received panobinostat and bortezomib as cited above. Treatment was continued for 3 weeks, and tumor volumes and survival of mice were recorded for each group. Mice were humanely sacrificed when the tumor volume reached 2,000 mm³. The day of death was noted and plotted on a Kaplan-Meier plot as described previously (14).

**Statistical analyses**

Data were expressed as mean ± SD. Comparisons used Student's t test or ANOVA, as appropriate. P values of <0.05 were assigned significance.

**Results**

**Panobinostat induces hyperacetylation and inhibition of hsp90 in MCL cells**

First, we confirmed whether panobinostat inhibits HDAC6 and induces hyperacetylation of hsp90 in MCL cells, as has been described in human leukemia cells (15). Figure 1A shows that treatment with panobinostat (20 nmol/L) inhibited the in vitro activity of recombinant HDAC6 by greater than 90% in a cell-free assay, which was only slightly augmented after exposure to 50 nmol/L panobinostat (Fig. 1A). Treatment with panobinostat, or knockdown of HDAC6 with siRNA, also induced hyperacetylation of hsp90 in JeKo-1 cells, indicative of intracellular inhibition of HDAC6 (Fig. 1B). This was further substantiated by the notable induction of α-tubulin acetylation in panobinostat-treated JeKo-1 cells (data not shown; refs. 15, 16). Panobinostat-induced hyperacetylation of hsp90 was associated with decreased binding of ATP to hsp90, which was previously reported by us to undermine the chaperone function of hsp90 (Fig. 1C; ref. 15). Consequently, treatment with panobinostat results in an increase in the levels of misfolded, polyubiquitylated hsp90 client proteins (15). Similarly, treatment with bortezomib inhibits the degradation of misfolded polyubiquitylated proteins by the proteasomes (18). Therefore, we determined the levels of polyubiquitylated proteins in JeKo-1 cell lysates treated with panobinostat and bortezomib. Although not as much as that following an exposure...
to 20 nmol/L of bortezomib, panobinostat (20 nmol/L) also resulted in accumulation of polyubiquitylated, especially high molecular weight, proteins in MCL cells (Fig. 1D). Collectively, these findings indicate that panobinostat-mediated hyperacetylation and inhibition of hsp90 leads to increased levels of polyubiquitylated proteins.

**Panobinostat abrogates bortezomib-induced aggresome formation in MCL cells**

HDAC6 has been shown to bind and sequester intracellular polyubiquitylated proteins into aggresome, which is a perinuclear organelle formed as a protective response against proteotoxic stress (e.g., induced by the proteasome inhibitor bortezomib; ref. 18). Therefore, we determined the effect of panobinostat on bortezomib-induced aggresome formation in MCL cells, using immunofluorescence and electron microscopy. As shown in Fig. 2A and B, treatment of JeKo-1 and Granta-519 cells with bortezomib led to formation of aggresome in up to 30% to 35% cells (Fig. 2A and B). Cotreatment with panobinostat abrogated bortezomib-induced aggresome formation (Fig. 2A and B; P ≤ 0.005 for bortezomib treatment versus the cotreatment).

Similar effects were observed following treatment with tubacin (5.0 μmol/L), which is a relatively specific but weak inhibitor of HDAC6 (data not shown). Transmission electron microscopy of JeKo-1 cells following exposure to bortezomib revealed the presence of electron-dense perinuclear aggresome (Fig. 2C), lacking a membrane envelope and surrounded by mitochondria (28). Panobinostat treatment alone induced ER dilation in JeKo-1 cells, indicative of ER...
stress, whereas combined treatment with panobinostat and bortezomib induced highly dilated ER and partial dissolution of the nuclear envelope (arrowhead; Fig. 2C, inset). This indicated the induction of a more profound ER stress. These data clearly show for the first time that treatment with panobinostat inhibits bortezomib-induced aggresome formation in human MCL cells.

**Panobinostat depletes hsp90 client proteins as well as induces ER stress and apoptosis in MCL cells**

Following panobinostat treatment, induction of ER stress in MCL cells was shown by the induction of the mRNA and protein markers of ER stress. Treatment with panobinostat induced XBP1s, Erdj4 (an ER homologue of hsp40 and a downstream target of XBP1s), and NOXA.

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**Fig. 2.** Panobinostat abrogates bortezomib-induced aggresome formation in JeKo-1 and Granta-519 cells. A, JeKo-1 cells were treated with bortezomib (BZ), panobinostat (PS), and a combination of the two (PS + BZ) for 16 hours. Cells were then stained for HDAC6 and ubiquitin. Then, immunofluorescence microscopy was done using LSM-510 confocal microscope using a 63×/1.2 W correction objective. B, JeKo-1 and Granta-519 cells were treated with bortezomib, panobinostat, or a combination of the two drugs. The percentage of cells with aggresomes was counted. Values were plotted as the percentage of aggresome-positive cells ± SD. *P < 0.005, bortezomib versus cotreatment. C, JeKo-1 cells were treated with the indicated doses of panobinostat and bortezomib, and the ultrastructure of cells was imaged using a transmission electron microscope. Aggresomes and ER dilation are indicated by arrows and depicted as insets.
mRNA more in JeKo-1 cells than in Z138C cells. CHOP mRNA was induced in both Z138C and JeKo-1 cells (Fig. 3A). Additionally, panobinostat treatment resulted in increased expression of CHOP, ATF4, and GRP78 in JeKo-1 and Z138C cells (Fig. 3B). Treatment with panobinostat led to a dose-dependent increase in apoptosis of JeKo-1, MO2058, Granta-519, and Z138C cells, as assessed by Annexin V and PI staining (Fig. 3C). This was associated with panobinostat-mediated depletion of hsp90 client proteins, including c-RAF, AKT, CDK4, and cyclin D1 (15, 17), as well as the induction of pro-apoptotic BAX and BAK and downregulation of survivin and c-Myc levels (Fig. 3D; ref. 29). These data show for the first time that treatment of MCL cells with clinically achievable concentration of panobinostat leads to UPR while simultaneously disrupting hsp90 chaperone function and depleting prosurvival and upregulating pro-apoptotic proteins. Consistent with the previous reports, our studies also show that treatment with bortezomib dose-dependently induced apoptosis in MO2058, Z138C, and JeKo-1 cells (Supplementary Fig. S1A). This was associated with bortezomib-induced ER stress as evidenced by induction of mRNA of XBP1s and its transcriptional target Erdj4. Bortezomib treatment also induced CHOP and NOXA mRNA levels (Supplementary Fig. S1B).

Fig. 3. Treatment with panobinostat induces ER stress, results in dose-dependent apoptosis, and abrogates hsp90 client proteins in MCL cells. A, JeKo-1 and Z18C cells were treated with the indicated doses of panobinostat for 6 hours, and the mRNA expression of XBP1s, XBP1u, Erdj4, CHOP, and NOXA was assessed by reverse transcription-PCR. The expression of β-actin served as a loading control. B, JeKo-1 and Z138C cells were treated with the indicated doses of panobinostat for 6 hours and the expression of ATF4, GRP78, and CHOP was assessed by immunoblotting. C, JeKo-1, MO2058, Z138C, and Granta-519 cells were exposed to the indicated doses of panobinostat for 48 hours, and apoptosis was monitored by Annexin V and PI staining followed by flow cytometry. D, JeKo-1 cells were exposed to indicated doses of panobinostat for 6 hours, and the expression of c-RAF, AKT, cyclin D1, CDK4, survivin, c-Myc, BAK, BAX, and β-actin were monitored by immunoblot analyses.
Cotreatment with panobinostat and bortezomib causes enhanced UPR in MCL cells

As suggested by the electron microscopy findings noted above, we next determined whether cotreatment with panobinostat would increase bortezomib-induced molecular hallmarks of UPR and ER stress. As shown in Fig. 4A, cotreatment with panobinostat increased bortezomib-induced GRP78, CHOP, PUMA, p-eIF2α, eIF2α, and GADD34 was assessed by immunoblot analysis. As demonstrated in Fig. 4A, the expression of CHOP, GADD34, ERO-1α, NOXA, and β-actin mRNA was assessed by reverse transcription-PCR following 6 hours of exposure of JeKo-1 cells to bortezomib and panobinostat. As compared with treatment with each

Fig. 4. Cotreatment of MCL cells with bortezomib and panobinostat induces enhanced ER stress in MCL cells. A, JeKo-1 cells were treated with indicated doses of panobinostat and/or bortezomib for 6 hours, and the expression of GRP78, CHOP, NOXA, PUMA, p-eIF2α, eIF2α, and GADD34 was assessed by immunoblot analysis. B, alternatively, the expression of CHOP, GADD34, ERO-1α, NOXA, and β-actin mRNA was assessed by reverse transcription-PCR following 6 hours of exposure of JeKo-1 cells to bortezomib and panobinostat. C, JeKo-1 cells were treated with panobinostat and bortezomib at a 1:1 molar ratio stained for Annexin V–PI. Percentage of apoptotic cells was assessed by flow cytometry. The combination index (CI) for each treatment was determined using Calcusyn software. Combination index values of less than 1.0 represent synergistic interactions. D, JeKo-1 cells were infected with retroviral scrambled siRNA or CHOP siRNA and treated with panobinostat following 48 hours of infection. Viability of cells following CHOP knockdown and 24 hours of exposure to panobinostat was assessed by trypan blue dye exclusion assay and hemocytometry. Values presented are an average of three independent experiments ± SD. Inset shows immunoblot analysis of CHOP protein after 48 hours of infection.

Cotreatment with panobinostat and bortezomib causes enhanced UPR in MCL cells

As suggested by the electron microscopy findings noted above, we next determined whether cotreatment with panobinostat would increase bortezomib-induced molecular hallmarks of UPR and ER stress. As shown in Fig. 4A, cotreatment with panobinostat increased bortezomib-induced GRP78, CHOP, and PUMA levels in JeKo-1 cells (Fig. 4A). Increased CHOP induction was also associated with the upregulation of the mRNA of another CHOP-inducible transcript, ERO-1α (Fig. 4B). Importantly, increased CHOP levels, following combined treatment with panobinostat and bortezomib, led to upregulation of GADD34 expression, a CHOP-inducible phosphatase responsible for dephosphorylating eIF2α (Fig. 4A; refs. 23–25, 30–32). This was associated with decreased phosphorylation of eIF2α after combined treatment with bortezomib and panobinostat. As compared with treatment with each
agent alone, cotreatment with panobinostat and bortezomib induced synergistic apoptosis of JeKo-1 cells, as indicated by the combination indices of <1.0 by isobologram analysis (Fig. 4C). The superior activity of the combination was also noted for Z138C cells (data not shown). Collectively, these observations also suggest that enhanced CHOP expression and ensuing CHOP-induced gene products may contribute to the lethal effects of panobinostat and bortezomib against MCL cells (Fig. 4B and C).

Effect of knockdown of CHOP on the sensitivity of MCL cells to panobinostat

We next determined the role of CHOP induction in the lethal UPR mediated by treatment with panobinostat or bortezomib. Retroviral knockdown of CHOP by siRNA partially decreased the sensitivity of JeKo-1 cells to panobinostat (P = 0.001; Fig. 4D). In contrast, knockdown of CHOP only slightly decreased the sensitivity of JeKo-1 cells to bortezomib-induced cell death (P > 0.05; data not shown). The modest effects of CHOP knockdown on bortezomib-induced cell death may be because treatment with bortezomib engages multiple mechanisms of cell death in transformed cells (8). For example, bortezomib treatment also induces the prodeath BH3-only domain containing the protein NOXA, as shown in Fig. 4A. Collectively, these observations indicate that CHOP induction contributes to panobinostat-induced lethal ER stress in MCL cells.

Cotreatment with bortezomib and panobinostat exerts superior in vitro activity against primary MCL cells and in vivo activity in mice with Z138C xenograft

The lethal effects of cotreatment with panobinostat and bortezomib were also determined against three samples of primary MCL cells (Table 1). As shown, each sample had different sensitivity to panobinostat- and bortezomib-mediated cell death, although cotreatment resulted in more cell death than treatment with each agent alone (P ≤ 0.05; Table 1). Due to inadequate number of cells in the MCL samples, full analysis of a synergistic effect of the combination could not be determined. However, compared with the effect of panobinostat or bortezomib alone, cotreatment with panobinostat and bortezomib for 24 hours caused more induction of GRP78, Erdj4, CHOP, and NOXA mRNA levels in the primary MCL samples (Fig. 5). As compared with each agent alone, cotreatment with panobinostat and bortezomib does not induce greater cell death or UPR in normal CD34+ bone marrow progenitor cells (Table 1; Fig. 5). To determine the in vivo effects of panobinostat and/or bortezomib, Z138C MCL cells were subcutaneously implanted in nonobese diabetic/severe combined immunodeficient mice. Tumors were allowed to grow until the average tumor volume reached 50 mm³. The mice were treated with vehicle, or with the previously determined submaximally tolerated dose of bortezomib (0.5 mg/kg once a week), panobinostat (10 mg/kg three times a week), or the combination of the two drugs. Tumor volumes were recorded, and the mice were humanely euthanized when the tumor volume reached 2,000 mm³. Following 3 weeks of treatment with panobinostat and/or bortezomib, the mean tumor volumes were significantly lower in mice treated with the combination, compared with those treated with each drug alone (P < 0.05; Fig. 6A). Moreover, treatment with panobinostat or the combination of bortezomib and panobinostat showed a significant increase in the survival of mice (P = 0.05 and 0.001, respectively) compared with treatment with bortezomib or vehicle alone (Fig. 6B). Median survival of mice treated with panobinostat and bortezomib was 47 days, compared with 41 and 37 days for the mice treated with panobinostat or vehicle alone, respectively.

Discussion

The present study shows for the first time that panobinostat inhibits HDAC6 in human MCL cells, thereby inducing hyperacetylation and attenuation of hsp90 chaperone function (15–17). Panobinostatin mediated HDAC6 inhibition also increases the intracellular levels of polyubiquitylated

**Table 1. Panobinostat and bortezomib induce cell death of primary MCL cells**

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<th>Sample</th>
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<th>25 nmol/L, BZ</th>
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<tr>
<td>Normal CD34+</td>
<td>2.8 ± 0.2</td>
<td>9.3 ± 3.8</td>
<td>30.1 ± 2.0</td>
<td>33.5 ± 3.0</td>
</tr>
<tr>
<td>MCL#1</td>
<td>2.7 ± 0.2</td>
<td>5.4 ± 0.3</td>
<td>10.1 ± 1.6</td>
<td>25.6 ± 1.7*</td>
</tr>
<tr>
<td>MCL#2</td>
<td>4.9 ± 2.0</td>
<td>27.7 ± 4.1</td>
<td>41.5 ± 1.2</td>
<td>51.5 ± 1.4*</td>
</tr>
<tr>
<td>MCL#3</td>
<td>9.4 ± 0.5</td>
<td>23.8 ± 1.0</td>
<td>25.1 ± 1.7</td>
<td>64.9 ± 2.5†</td>
</tr>
</tbody>
</table>

NOTE: Primary MCL cells from three patients and normal CD34+ cells were treated in triplicate for 48 hours with the indicated doses of panobinostat and bortezomib. The average viability of cells was then assessed through trypan blue dye exclusion and hemocytometry. Values represent the percentage of dead cells ± SD from each condition compared with untreated cells. Abbreviations: BZ, bortezomib; PS, panobinostat.

*P ≤ 0.05.
†P ≤ 0.005.
proteins because HDAC6 has been shown to regulate the accumulation of ubiquitylated, toxic protein aggregates and noted to be a cellular stress surveillance factor (33, 34). While increasing the intracellular accumulation of misfolded proteins, treatment with panobinostat simultaneously abrogates HDAC6-mediated aggresome formation, which is normally an adaptive and protective response—the first line of defense against misfolded proteins (18). Collectively, as underscored by these findings, panobinostat-induced perturbations in protein triage result in UPR and ER stress in MCL cells. This is associated with increased levels of XBPs, as well as activation of PERK and p-eIF2α. The latter is designed to reduce general protein synthesis while concomitantly promoting protein folding through increased levels of the molecular chaperones, such as GRP78, as well as enhancing the ER-associated protein degradation (22, 23). However, sustained exposure to panobinostat and induction of CHOP leads to apoptosis, thus converting the protective and adaptive response to a lethal UPR in MCL cells.

The role of a maintenance or “physiologic UPR” has been well appreciated in B-cell development and maturation. For instance, IRE1α has been reported to be crucial for B lymphopoiesis, IgG rearrangement, and production of B-cell receptors (35). Induction of XBPs mRNA is established as an important step in B-cell maturation into plasma cells, and XBPs expression is known to drive multiple myeloma (MM) pathogenesis (36, 37). Therefore, the significance of ER stress in B-cell development, maturation, and function also creates the rationale as to why transformed B cells might be especially susceptible to agents that accentuate the UPR, leading to protracted and lethal ER stress. This approach has been clinically validated and exemplified by the anti-MCL and anti-MM activity of bortezomib, which induces lethal UPR in the preclinical models of these malignancies (6, 38). Present studies, however, show the mechanistic link between panobinostat-mediated increased accumulation of polyubiquitylated proteins and HDAC6 inhibition and aggresome disruption to panobinostat-mediated accentuation of bortezomib-induced proteotoxic stress and lethal UPR in human MCL cells. These findings are consistent with the previous reports demonstrating that HDI treatment induced UPR in MM cells, and cotreatment with...
panobinostat further enhanced bortezomib-induced UPR and apoptosis of MM cells (38, 39). Our results presented here show that cotreatment with bortezomib seems to further heighten the sensitivity of MCL cells to panobinostat-induced aggresome dysfunction and also to heighten UPR. Catley et al. reported a similar effect in MM cells (40). In MCL cells, compared with treatment with panobinostat or bortezomib alone, cotreatment with panobinostat and bortezomib led to synergistic and lethal ER stress, as evidenced by ultrastructural changes showing dilated ER, as well as by increased levels of CHOP and NOXA. Previous studies have also documented a mechanistic role of increased CHOP and NOXA levels underlying lethal ER stress (30, 41, 42). Collectively, the significance of these findings is highlighted in the model presented in Fig. 7. Furthermore, in support of the model, our findings show that knockdown of CHOP confers resistance to panobinostat-induced cell death in MCL cells. In contrast, CHOP knockdown did not significantly reduce bortezomib-induced cell death. This may be because bortezomib activates multiple mechanisms and is consequently less dependent on CHOP in mediating its lethal effects in MCL cells (8). Although not seen in the cultured MCL cells used in the present studies, increased BIM levels have also been shown to mediate the lethal outcome of UPR (43).

Our findings also show that, in MCL cells, in addition to upregulating prodeath proteins, panobinostat-mediated hyperacetylation of hsp90 and attenuation of hsp90 chaperone function are also associated with depletion of important progrowth and prosurvival, MCL-relevant oncoprotein client proteins, including cyclin D1, CDK4, MYC, AKT, and c-RAF. Most likely, this also lowers the threshold for apoptosis in MCL cells (17, 29). Additionally, in previous studies, both panobinostat and bortezomib have been shown to induce reactive oxygen species, which may contribute to the cytotoxicity of the combination in MCL cells (7, 8, 14). The synergism observed with the combination of HDAC inhibitors and bortezomib could also be, at least partly, due to inhibition of NF-κB induction by bortezomib because treatment with panobinostat alone is known to induce NF-κB, which may exert an antiapoptotic effect (44). Taken together, these observations indicate that in addition to lethal UPR, other mechanisms may be involved in the observed synergistic activity of panobinostat and bortezomib in MCL cells, as is proposed in the model presented in Fig. 7. The combination of panobinostat and bortezomib also seems to hasten the built-in negative feedback mechanism in UPR to dephosphorylate eIF2α by inducing GADD34 (31, 32). This is probably a direct consequence of CHOP upregulation and activation of its downstream target GADD34 or the stabilization of GADD34 by proteasome inhibition (31, 32, 45).

Results presented here show that compared with each agent alone, the combination of panobinostat and bortezomib also showed superior activity against primary MCL cells. Although full evaluation of the molecular correlates of this superior activity was not feasible, its molecular correlates included induction of mRNA levels of CHOP and NOXA, suggesting increased and lethal ER stress in the primary MCL cells. More complete analyses of the effects of panobinostat and bortezomib have to be conducted before any firm conclusions can be drawn about the degree of superior activity and about the predictive biomarkers of response to the combination. Cotreatment with panobinostat and bortezomib also resulted in increased tumor growth delay and
survival in a mouse xenograft model of human MCL cells. Recently, results of a phase I study of a combination of vorinostat and bortezomib showed that the combination of drugs belonging to these two classes of agents is clinically feasible and shows promising clinical activity in bortezomib refractory MM (46). Supported by our preclinical in vitro and in vivo findings presented here, the results of this trial support the rationale for testing the combination of panobinostat and bortezomib in patients with MCL.

Disclosure of Potential Conflicts of Interest

K.N. Bhalla, commercial research grant, speaker’s bureau, Novartis; advisory board, Novartis, Merck.

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Role of CAAT/Enhancer Binding Protein Homologous Protein in Panobinostat-Mediated Potentiation of Bortezomib-Induced Lethal Endoplasmic Reticulum Stress in Mantle Cell Lymphoma Cells


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