

Treatment with Panobinostat Induces Glucose-Regulated Protein 78 Acetylation and Endoplasmic Reticulum Stress in Breast Cancer Cells

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Abstract

Increased levels of misfolded polypeptides in the endoplasmic reticulum (ER) triggers the dissociation of glucose-regulated protein 78 (GRP78) from the three transmembrane ER-stress mediators, i.e., protein kinase RNA-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme 1 α , which results in the adaptive unfolded protein response (UPR). In the present studies, we determined that histone deacetylase-6 (HDAC6) binds and deacetylates GRP78. Following treatment with the pan-histone deacetylase inhibitor panobinostat (Novartis Pharmaceuticals), or knockdown of HDAC6 by short hairpin RNA, GRP78 is acetylated in 11 lysine residues, which dissociates GRP78 from PERK. This is associated with the activation of a lethal UPR in human breast cancer cells. Coimmunoprecipitation studies showed that binding of HDAC6 to GRP78 requires the second catalytic and COOH-terminal BUZ domains of HDAC6. Treatment with panobinostat increased the levels of phosphorylated-eukaryotic translation initiation factor (p-eIF2 α), ATF4, and CAAT/enhancer binding protein homologous protein (CHOP). Panobinostat treatment also increased the proapoptotic BIK, BIM, BAX, and BAK levels, as well as increased the activity of caspase-7. Knockdown of GRP78 sensitized MCF-7 cells to bortezomib and panobinostat-induced UPR and cell death. These findings indicate that enforced acetylation and decreased binding of GRP78 to PERK is mechanistically linked to panobinostat-induced UPR and cell death of breast cancer cells. *Mol Cancer Ther*; 9(4); 942–52. ©2010 AACR.

Introduction

Glucose-regulated protein 78 (GRP78) or BiP (immunoglobulin heavy chain binding protein) is an endoplasmic reticulum (ER)-resident, ATP-dependent homologue of the molecular chaperone heat shock protein 70 (hsp70; ref. 1). GRP78 is primarily involved in the folding and assembly of newly synthesized polypeptides in the ER and chaperoning of improperly folded proteins (1, 2). In a process also referred to as ER-associated degradation, GRP78 recognizes hydrophobic regions of ER-associated degradation substrates and retains them in a soluble conformation thus preventing their aggregation (1–3). ATP hydrolysis releases the misfolded proteins from GRP78, which are then retrotranslocated into the cytosol and degraded by the proteasome (1–3). GRP78 also serves as a

master regulator of ER stress response, also called unfolded protein response (UPR), that can ensue following several forms of cellular stress, e.g., alterations in Ca²⁺ homeostasis, inhibition of protein glycosylation, or alterations in the redox status which compromise ER protein folding capacity and results in the accumulation of misfolded proteins (4, 5). In unstressed cells, GRP78 binds to the luminal domain of the three documented ER transmembrane receptors (mediators of UPR), i.e., inositol-requiring enzyme 1 α (IRE1 α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor-6 α (ATF6 α) and keeps them inactive (4, 5). Accumulation of misfolded proteins in the ER leads to the dissociation of GRP78 from the three receptors, leading to their activation (4, 5). Activated PERK phosphorylates eIF2 α (eukaryotic initiation factor) which blocks “cap-dependent” protein synthesis but results in the preferential translation of ATF4, a transcription factor that upregulates the proapoptotic transcription factor CHOP (CAAT/enhancer binding protein homologous protein) and genes required to restore normal ER function (4, 5). Activated IRE1 catalyzes the splicing of XBP1 to generate a frame-shift splice variant of XBP1, called XBP1s, that transactivates and upregulates GRP78, which participates in restoring normal ER function (4–6). However, protracted ER stress results

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Editor's Note: A question was raised regarding the content of a figure in this article. The editors reviewed this inquiry in 2019 and determined that no action was needed at that time.

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doi: 10.1158/1535-7163.MCT-09-0988

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in sustained activation of eIF2 α phosphorylation and CHOP induction, resulting in a lethal outcome for the UPR (7). Unresolved and protracted ER stress–triggered apoptosis involves the induction of BH3 domain-only proteins BIK and BIM (8, 9), as well as the activation of the intrinsic pathway of apoptosis involving the activity of caspase-9, caspase-3, and caspase-7 (10, 11). Intracellular proteotoxic stress and UPR is a common accompaniment of cancer cell transformation (12–14). Consistent with this, elevated levels of protective GRP78 have been shown to have an important role in the pathogenesis of several types of cancers, in which it confers poor prognosis (15, 16). Conversely, homozygous deletion of GRP78 in prostate epithelium inhibits prostate tumorigenesis (17). GRP78 inhibits cell death by inhibiting caspase-induced apoptosis in cancer cells (18–20). Related to this, knockdown of GRP78 has been documented to induce apoptosis and sensitize a variety of cancer cells to anti-cancer drug–induced apoptosis (18, 20).

Although the role of GRP78 as a regulator of the cellular ER stress response is well recognized, intrinsic post-translational modifications regulating GRP78 chaperone function and dissociation from the mediators of UPR (PERK, ATF6, and IRE) have not been well characterized. Specifically, the effect of acetylation of lysine residues on the function of GRP78 has not been determined, compared with other molecular chaperones such as hsp90 and hsp70, which have been previously described (21–23). Several studies, including our own, have shown that the predominantly cytosolic histone deacetylase-6 (HDAC6) is the deacetylase for hsp90 and hsp70 (21–23). Inhibition of activity of HDAC6 by pan-HDAC inhibitors, or by knockdown of HDAC6, leads to hyperacetylation of hsp90 (21–23). This attenuates ATP and cochaperone binding of hsp90, and inhibits its chaperone function toward its client proteins (21–24). Additionally, HDAC6 binds to misfolded, polyubiquitylated proteins through its BUZ (binding to ubiquitin zinc finger) domain and shuttles them into protective, perinuclear structures called aggresomes (25). Several studies have highlighted HDAC6 as an important cellular proteotoxic stress surveillance factor and a regulator of the cellular UPR induced by environmental, physiologic, or pathologic stress (26–28).

Based on the function of GRP78 as a molecular chaperone involved in alleviating ER stress due to misfolded proteins in the ER, as well as on the newly recognized role of HDAC6 in stress surveillance, we determined whether HDAC6 is the deacetylase for GRP78 and whether treatment with the pan-HDAC inhibitor panobinostat, which has been shown to induce the hyperacetylation and inhibition of the chaperone function of hsp90 and hsp70, would also exert a similar effect on GRP78 in the ER and induce UPR. Our findings show that HDAC6 is a GRP78 deacetylase. Inhibition of HDAC6 by panobinostat or knockdown of HDAC6 induces acetylation of GRP78 in human breast cancer cells. These findings also show that panobinostat-induced acetylation of GRP78 is

associated with reduced binding of GRP78 to PERK, increased levels of p-eIF2 α , ATF4, and CHOP in human breast cancer cells. Additionally, knockdown of GRP78 with short hairpin RNA (shRNA) sensitizes breast cancer cells to panobinostat-induced as well as to the proteasome inhibitor bortezomib-induced UPR and cell death.

Materials and Methods

Reagents and antibodies

Panobinostat was provided by Novartis Pharmaceuticals, Inc. Anti-hsp90 antibody was purchased from StressGen Biotechnologies, Corp. Monoclonal anti-acetyl lysine antibody was purchased from Cell Signaling Technology. Monoclonal anti-acetyl α -tubulin, anti-FLAG M2, and anti- β -actin antibodies were purchased from Sigma Aldrich Corporation. HDAC6, GRP78, PERK, ATF4, and CHOP antibodies were purchased from Santa Cruz Biotechnology, Inc. Phosphorylated eIF2 α and eIF2 α were obtained from Cell Signaling Technology. FLAG-tagged HDAC6 (wild-type) was a gift from Dr. Ed Seto (H. Lee Moffitt Cancer Center, Tampa, FL) and the FLAG-H216A (first catalytic domain mutant), FLAG-double mutant (catalytically inactive mutant), and Δ BUZ mutant HDAC6 constructs were a kind gift from Dr. Tso Pang Yao (Duke University, Durham, NC). FLAG-H611A (second catalytic domain mutant) was created by using the Quick-Change kit from Stratagene.

Cell culture

MCF-7, MDA-MB-231, and HEK-293T cells were obtained from American Tissue Culture Collection and cultured as recommended and previously described (22, 28). Logarithmically growing cells were exposed to the designated concentrations and exposure interval of the drugs. Following these treatments, cells were washed free of the drug(s) using PBS, and pelleted prior to performing further studies described below.

Western blot analyses and immunoprecipitation

Western blot analyses of GRP78, p-eIF2 α , eIF2 α , ATF4, CHOP, HDAC6, and β -actin were done using specific antibodies, as described previously (23, 29, 30). The expression of β -actin was used as a loading control. Immunoprecipitation of HDAC6, endogenous GRP78, and FLAG-tagged GRP78 were carried out using specific antibodies as previously described and immunoblotted with anti-GRP78, HDAC6, PERK, or anti-acetyl lysine antibody (30). Class-specific IgG was used as a control in immunoprecipitations. Horizontal scanning densitometry was done on Western blots by using acquisition into Adobe PhotoShop (Adobe Systems, Inc.) and analysis by the NIH Image Program (U.S. NIH, Bethesda, MD).

Transfections

MCF-7, MDA-MB-231, and HEK-293T cells were transiently transfected according to the instructions of the manufacturer using Lipofectamine and Plus

Reagent (Invitrogen) with plasmids containing scrambled oligonucleotide (control shRNA) or shRNA to GRP78 (SuperArray Bioscience Corporation) containing a 21-nucleotide sequence, corresponding to GRP78 mRNA—5'-GCAACTGGTTAAAGAGTTCTT-3'. For HDAC6 knockdown, HEK-293T cells were transfected with pBS/U6 (control vector) with or without the HDAC6 small interfering RNA (siRNA), which has a 21-nucleotide sequence 5'-GG ATG GAT CTG AAC CTT GAG A-3', corresponding to the targeted nucleotides 200 to 219 in the HDAC6 mRNA (accession no. BC013737; ref. 24). Knockdown of SIRT2 was carried out using control shRNA containing scrambled nucleotides or shRNA to SIRT2 containing the sequence 5'-GAAACATCCGGAACCCTTC-3' corresponding to SIRT2 mRNA.

Confocal microscopy

MCF-7 cells were labeled with immunofluorescence-tagged antibodies, as previously described (23). Briefly, MCF-7 cells were cultured on chamber slides, fixed with 4% paraformaldehyde for 10 min, washed, and then incubated with 0.5% Triton-PBS buffer for 5 min. The slides were washed, blocked with 3% bovine serum albumin and then stained with HDAC6 (Santa Cruz Biotechnology), GRP78 (Thermo Fisher Scientific), FLAG M2, and calnexin (Abcam) antibodies for 2 h. Following washes with PBS, fluorescently tagged anti-rabbit Alexa Fluor 488 or 594 and anti-mouse Alexa Fluor 594 or 488 (Invitrogen) as appropriate, were applied onto the slides for 1 h and washed with PBS. Coverslips were mounted on slides with Vectashield-DAPI mountant (Vector Laboratories) and the slides were visualized under Zeiss LSM-510 metaconfocal microscope. Images were captured at 63× magnification.

Purification of acetylated GRP78

Using anti-FLAG M1 affinity beads, FLAG-tagged GRP78 protein was affinity-captured from FLAG-GRP78-transfected HEK-293T cells that had been treated with 100 nmol/L of panobinostat (Novartis Pharmaceuticals, Inc.) as described previously for hsp90α acetylation (22). This was followed by immunoprecipitation of acetylated FLAG-tagged GRP78 using acetyl lysine agarose beads. The immunoprecipitated proteins were resolved by 8% SDS-PAGE gel and visualized using Coomassie blue stain. The band corresponding to GRP78 was excised, digested with trypsin, and the acetylated sites in the resulting tryptic digests were determined by mass spectrometry at the Proteomics Core Facility of the Medical College of Georgia, Augusta, GA (22).

Reverse transcription-PCR

Total RNA was isolated from MCF-7 cells using RNeasy RNeasy-4PCR kit Applied Biosystems according to the instructions of the manufacturer. Reverse transcription was done with 2 μg of RNA using Superscript RT

(Invitrogen). PCR reactions were done using the listed specific primers using 2× Supermix IQ (Bio-Rad) reagent, as previously described (30). The identity of PCR products was confirmed by sequencing. Primers sequences used for RT-PCR are as follows:

XBP1s	Forward primer	TCTGCTGAGTCCGCAGCAG
	Reverse primer	GAAAAGGGAGGCTGGTAAGGAAC
XBP1u	Forward primer	GAGATGTTCTGGAGGGGTGACA- AGTG
	Reverse primer	TGGTTGCTGAAGAGGAGGCCGGAAG

Statistical analyses

Data were expressed as mean ± SEM. Comparisons used Student's *t* test. *P* < 0.05 values were assigned significance.

Results

Inhibition of HDAC6 activity induces acetylation of GRP78

In previous studies, we documented the role of HDAC6 in deacetylating hsp90 and hsp70, as well as showing that inhibition of HDAC6 by panobinostat or knockdown of HDAC6 induces acetylation of hsp90 and hsp70 (22, 23). In the present studies, we determined that exposure to clinically achievable levels of panobinostat also induced acetylation of the endogenous GRP78 in human breast cancer MCF-7 and MB-231 cells (Fig. 1A). We also observed that treatment with panobinostat reduced HDAC6 but increased GRP78 levels, consistent with panobinostat-mediated inhibition of the chaperone function of hsp90 and induction of ER stress response, as has been documented for other HDAC inhibitors (31). Panobinostat treatment also dose-dependently induced acetylation of the ectopically expressed FLAG-GRP78 in HEK-293T cells (Fig. 1B). Ectopic expression of HDAC6 siRNA (3 μg) into HEK-293T cells lowered HDAC6 levels by ~60%, which was associated with the induction of GRP78 acetylation, which was also seen following treatment with panobinostat (Fig. 1C). As has been previously shown, *in vitro* and *in vivo* knockdown of HDAC6 also induced acetylation of α-tubulin (Fig. 1C; refs. 32–34). Taken together, these findings indicate that HDAC6 is also a deacetylase for GRP78. Next, we determined the identity of the acetylated lysine residues in GRP78 induced by panobinostat. HEK-293T cells transfected with GRP78 were treated with panobinostat, and the acetylated GRP78 was affinity-immunopurified using anti-FLAG conjugated M2 agarose, followed by agarose beads bearing immobilized anti-acetyl lysine antibody. The enriched acetylated GRP78 was analyzed by nano-high performance liquid chromatography/mass spectrometry/mass spectrometry in a mass spectrometer (22).

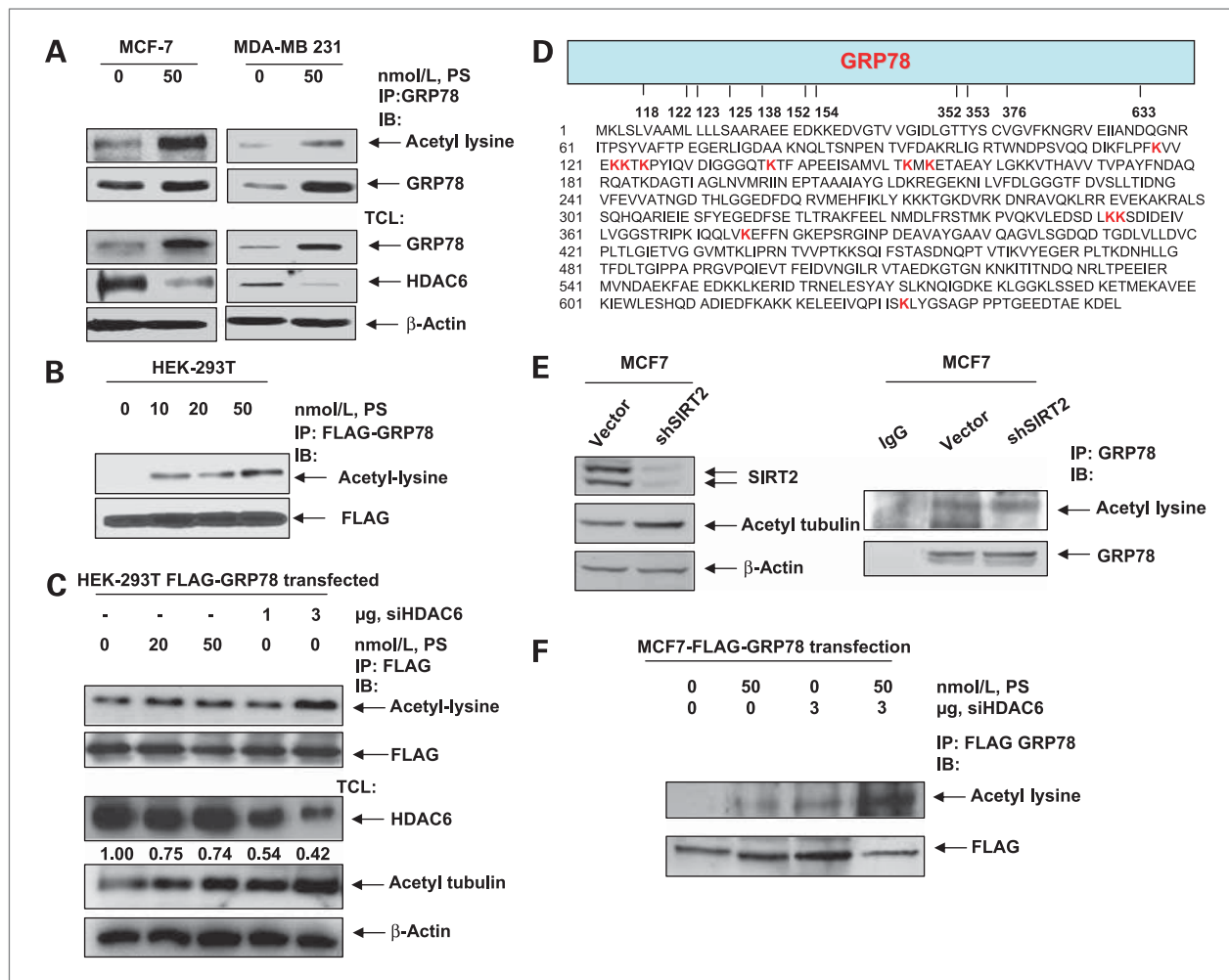


Figure 1. Panobinostat induces GRP78 acetylation and HDAC6 is the GRP78 deacetylase. **A**, MCF-7 and MDA-MB-231 cells were exposed to the indicated dose of panobinostat (PS) and the cells were washed free of the drug. The pellets were lysed and GRP78 was immunoprecipitated (IP) from the lysates followed by immunoblotting (IB) with acetyl-lysine antibody. Alternatively, the lysates were immunoblotted with GRP78, HDAC6, and β -actin. **B**, HEK-293T cells were transfected with FLAG-tagged GRP78 and exposed to panobinostat for 16 h. Acetylation of FLAG-tagged GRP78 was detected by immunoprecipitating FLAG from the lysate and immunoblotting for acetyl-lysine. **C**, HEK-293T cells were cotransfected with empty vector or siHDAC6 construct and incubated for 48 h. FLAG-tagged GRP78-transfected cells were exposed to the indicated doses of panobinostat for 16 h. The lysates were immunoprecipitated with FLAG antibody and then immunoblotted for anti-acetyl lysine to assess GRP78 acetylation. Alternatively, total lysates were immunoblotted for acetyl-tubulin, HDAC6, and β -actin. **D**, acetylated lysine residues identified by LC-MS/MS (red). **E**, MCF7 cells transfected with control shRNA or shRNA to SIRT2 were immunoblotted for SIRT2, acetyl-tubulin and β -actin. GRP78 was immunoprecipitated from control shRNA or shSIRT2-transfected cells and immunoblotted for acetyl-lysine to determine the effect of SIRT2 knockdown on the acetylation of GRP78. **F**, MCF7 cells were cotransfected with FLAG-tagged GRP78 and vector or siRNA to HDAC6 for 48 h and exposed to the indicated concentration of panobinostat for 16 h. Acetylation of FLAG-GRP78 was assessed by immunoprecipitating with FLAG antibody and immunoblotting for acetyl-lysine.

Eleven acetylated lysine residues were identified in GRP78. These include, K118, K122, K123, K125, K138, K152, K154, K352, K353, K376, and K633 (Fig. 1D). Because panobinostat also inhibits other lysine deacetylases, whether all of these lysine residues are deacetylated solely by HDAC6 remains unclear. Figure 1E shows that, unlike HDAC6, SIRT2 is not a deacetylase for GRP78, because shRNA mediated knockdown of SIRT2 did not increase the acetylation of GRP78. We chose to examine the role of SIRT2 because like HDAC6, SIRT2 is also predominantly cytosolic and is a deacetylase for α -tubulin. We also determined whether the acetylation of GRP78 was higher in cells treated with panobinostat, as compared with HDAC6 knockdown alone. Figure 1F shows that treatment with 50 nmol/L of panobinostat, which is known to decrease HDAC6 activity by \sim 90%, induces as much GRP78 acetylation as is seen following \sim 60% knockdown of HDAC6 (3 μ g of siRNA). Cotreatment with 50 nmol/L of panobinostat induced more GRP78 acetylation in cells with knockdown of HDAC6 (also with 3 μ g of siRNA). Increased GRP78 acetylation observed with near-complete inhibition

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of the activity of HDAC6 following treatment of cells combined with HDAC6 knockdown with 50 nmol/L of panobinostat suggests that HDAC6 is the predominant deacetylase for GRP78. However, our data does not exclude the unlikely possibility that a separate cytosolic class II HDAC might be contributing to the deacetylation of GRP78.

HDAC6 interacts with GRP78 through its second catalytic domain and BUZ domain

Next, we determined the domain by which HDAC6 binds to GRP78. First, we showed that in MCF-7 and

MDA-MB-231 cell lysates, GRP78 could be reversibly coimmunoprecipitated with the endogenous HDAC6 (Fig. 2A). Furthermore, we transfected the FLAG-tagged constructs of the full-length, wild-type HDAC6 with the intact two catalytic deacetylase domains (I and II), and the previously described mutants of the catalytic domain I (H216A) and/or catalytic domain II (H611A), or the BUZ domain deleted (Δ BUZ) mutant of HDAC6 (as outlined in Fig. 2B) into HEK-293T cells, and determined the binding of the anti-FLAG antibody immunoprecipitates to GRP78 (21, 25). Figure 2C shows that an intact catalytic domain II and the BUZ domain of

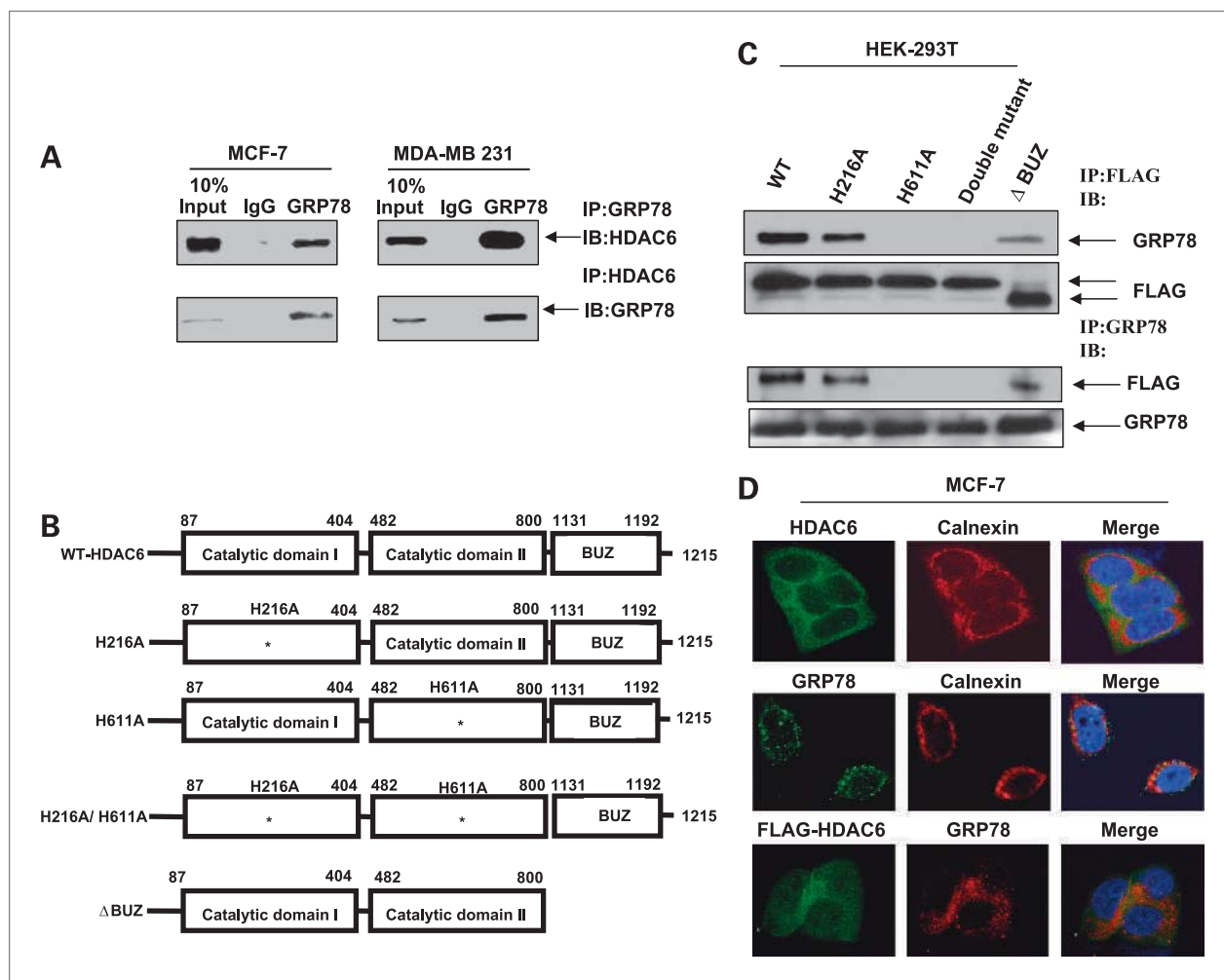


Figure 2. GRP78 interacts with HDAC6. **A**, MCF-7 and MDA-MB-231 cells were lysed and GRP78 was immunoprecipitated from the lysates. This was followed by immunoblotting with anti-HDAC6 antibody. Alternatively, HDAC6 was immunoprecipitated from MCF-7 and MDA-MB-231 and the immunoprecipitates were immunoblotted for GRP78. **B**, domain structure of human HDAC6: human HDAC6 comprises two catalytic domains and a BUZ domain. H216A mutation renders the catalytic domain 1 inactive whereas H611A renders the catalytic domain 2 inactive and the double mutant is catalytically inactive. The Δ BUZ mutant is deleted from the BUZ domain, which interacts with polyubiquitinated proteins. **C**, HEK-293T cells were transfected with either FLAG-tagged wild-type HDAC6 (WT-HDAC6), H216A mutant, H611A mutant, double mutant, or Δ BUZ constructs and the lysates were immunoprecipitated with FLAG antibody. **C**, the interaction of GRP78 with transfected constructs was analyzed by immunoblotting for GRP78. Reverse immunoprecipitations were performed to confirm the findings. **D**, MCF-7 cells were fixed and stained for HDAC6 and calnexin, GRP78 and calnexin, or GRP78 and FLAG-HDAC6 antibodies. The colocalization of HDAC6 with ER was visualized by confocal immunofluorescent microscopy. Merged images demonstrate the colocalization of HDAC6 and GRP78 with calnexin (an ER membrane marker).

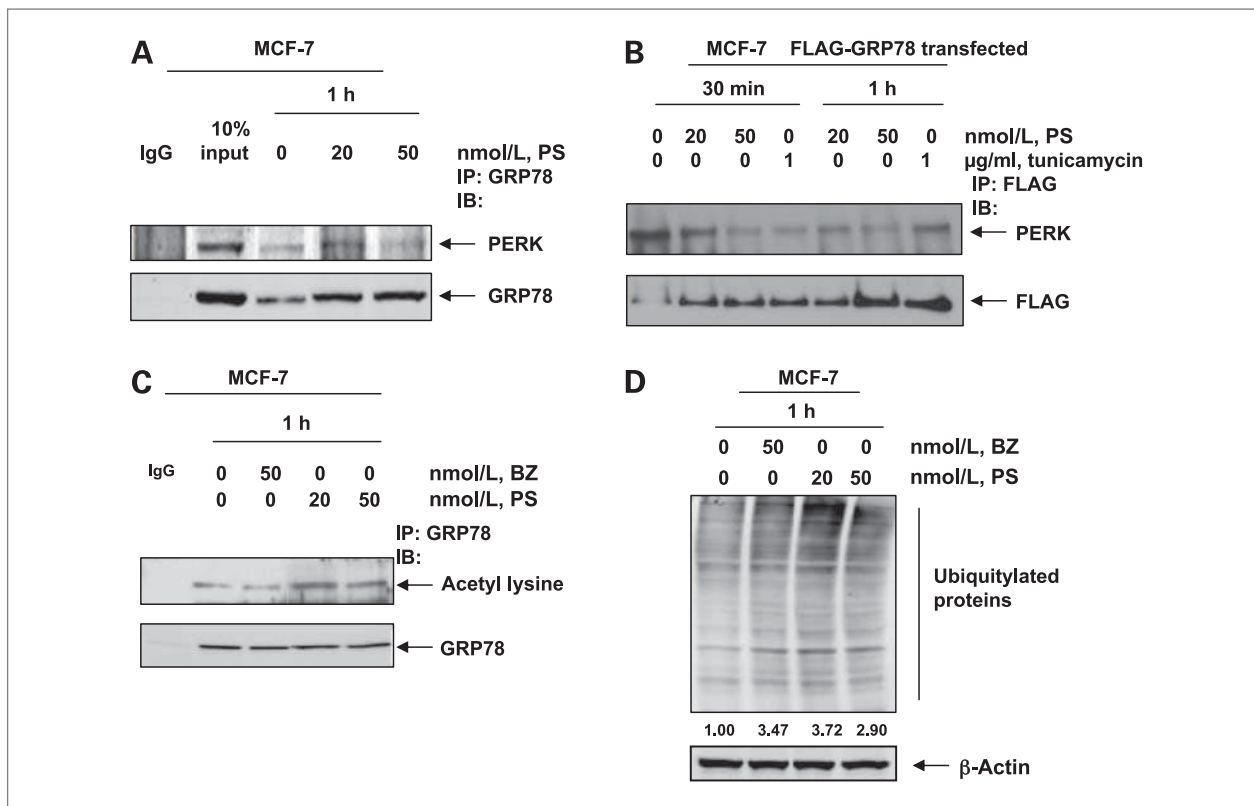


Figure 3. Acetylation of GRP78 results in a decreased binding of PERK to endogenous and FLAG-tagged GRP78. **A**, MCF-7 cells were exposed at the indicated times and exposure of panobinostat and GRP78 was immunoprecipitated from the resulting lysates. The immunoprecipitates were immunoblotted for PERK and GRP78. **B**, MCF-7 cells were transfected with FLAG-tagged GRP78 and exposed to the indicated doses of tunicamycin and panobinostat for 30 min and 1 h. Subsequently, the lysates from the cell pellets were immunoprecipitated with anti-FLAG antibody and immunoblotted for PERK and FLAG. **C**, MCF-7 cells were exposed to bortezomib and panobinostat for 1 h and GRP78 was immunoprecipitated from the resulting lysates. Acetylation of GRP78 was assessed by immunoblotting for acetyl lysine. **D**, alternatively, the lysates were immunoblotted for polyubiquitin to detect ubiquitylated proteins.

HDAC6 were essential for the interaction of GRP78 with HDAC6. The H611A mutant and the fully catalytically inactive H216A/H611A mutant forms of HDAC6 did not show any binding to GRP78, whereas the Δ BUZ mutant of HDAC6 minimally bound GRP78 (Fig. 2C). We further confirmed the interaction between GRP78 and HDAC6 at the ER by confocal immunofluorescence analysis using fluorescence-labeled anti-GRP78, anti-HDAC6, and anti-calnexin antibodies. Figure 2D shows that both the endogenous HDAC6 and GRP78 localize with calnexin, an ER-membrane protein, presumably at the rough ER which remains associated with the nucleus. Ectopically expressed FLAG-HDAC6 also colocalizes with GRP78 in the perinuclear ER location (Fig. 2D). Because for both GRP78 and HDAC6, only the rabbit polyclonal antibodies are reliable for the immunofluorescent analyses, we were unable to use these antibodies for showing the colocalization of the endogenous GRP78 and HDAC6 on the ER.

Panobinostat-induced acetylation of GRP78 is associated with decreased binding of GRP78 with the ER stress mediator PERK inducing ER stress response

In response to increasing levels of misfolded proteins in the ER, GRP78 has been documented to dissociate from the ER-bound mediators of UPR, i.e., PERK, ATF6, and IRE1 and switch its binding to the unfolded proteins (4, 5). We next determined whether panobinostat-induced GRP78 acetylation affects its binding to PERK in breast cancer cells. Following exposure to panobinostat for 60 min, binding of PERK to endogenous GRP78 was reduced in MCF-7 cells, even though panobinostat treatment increased the intracellular levels and acetylation of GRP78 (Fig. 3A and B). A rapid decline in the binding of ectopically expressed FLAG-GRP78 to PERK was also seen in MCF-7 cells, following treatment with panobinostat or tunicamycin, a classic ER stress inducer, for intervals as short as 30 min (Fig. 3C). During this interval, panobinostat and tunicamycin also

rapidly increased the levels of FLAG-GRP78 in MCF-7 cells. Again, a panobinostat-mediated decrease in the binding of GRP78 to PERK was associated with the induction of GRP78 hyperacetylation in MCF-7 cells (Fig. 3C). Consistent with these observations, similar to the proteasome inhibitor bortezomib, treatment with panobinostat for 60 min also increased the intracellular levels of polyubiquitylated proteins (Fig. 3D). Overall, these findings suggest that, in conjunction with increasing the levels of intracellular unfolded polyubiquitylated proteins, treatment with panobinostat induces GRP78 acetylation and impairs its ability to bind PERK.

Panobinostat-induced acetylation of GRP78 results in protracted UPR and cell death

We next determined the functional significance of panobinostat-induced acetylation and decreased binding

of GRP78 to PERK with respect to triggering lethal UPR in breast cancer cells. Although UPR is primarily a protective response against proteotoxic stress due to the accumulation of misfolded proteins in the ER, protracted ER stress and UPR could be lethal if the accumulated misfolded proteins cannot be cleared to restore homeostasis (5, 7). Consistent with previous reports, we also observed that exposure to bortezomib and panobinostat induced classical features of UPR. As shown in Fig. 4A, similar to bortezomib, treatment with panobinostat for 1 h increased PERK-mediated p-eIF2 α levels in MCF-7 cells (Fig. 4A). However, the total eIF2 α levels were not affected. Both bortezomib and panobinostat also induced the mRNA levels of XBP1s but not of XBP1u (Fig. 4B). Longer exposure intervals to panobinostat, similar to bortezomib, caused sustained increase in the levels of not only ATF4, GRP78, and its acetylated form (data

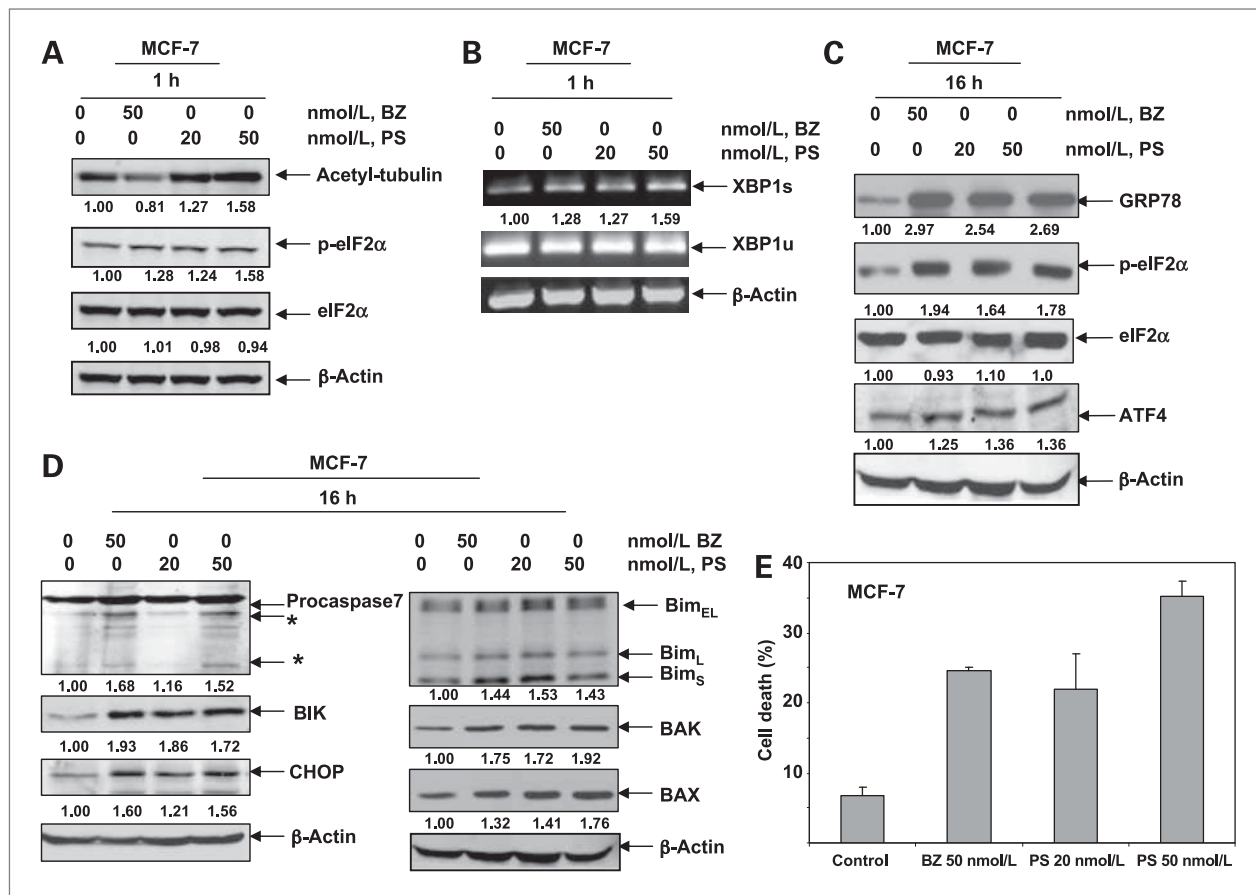
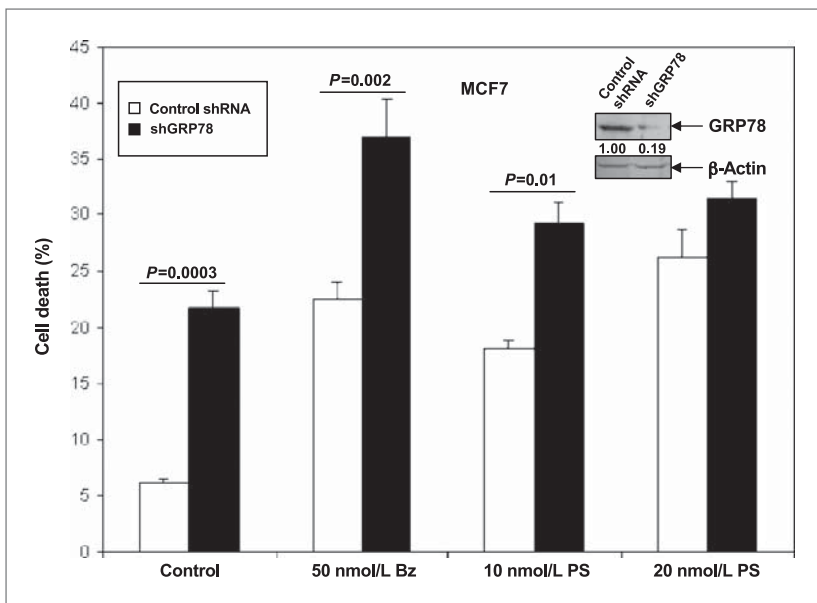


Figure 4. Panobinostat treatment induces GRP78 and an ER stress response in MCF-7 cells. A, MCF-7 cells were exposed to bortezomib and panobinostat for the indicated time points. Expression of acetyl-tubulin, p-eIF2 α , eIF2 α , and β -actin were assessed by immunoblotting. B, MCF-7 cells were exposed to panobinostat and bortezomib for 1 h, and the expression XBP1s, XBP1u, and β -actin mRNAs was monitored by RT-PCR. C and D, following exposure to bortezomib and panobinostat for 16 h, the expression of GRP78, p-eIF2 α , eIF2 α , ATF4, caspase-7, CHOP, BIK, Bim, Bax, Bak, and β -actin was assessed by immunoblot analyses. E, MCF-7 cells were exposed to panobinostat and bortezomib for 24 h and percentage of cell death was assessed by trypan blue staining.

Figure 5. Knockdown of GRP78 sensitizes MCF-7 to panobinostat-induced cell death: MCF-7 transfected with control shRNA or shGRP78 construct and following 24 h were exposed to the indicated-doses of bortezomib and panobinostat for an additional 24 h. Percentage cell death was assessed by trypan blue staining. Inset shows knockdown of GRP78.



not shown), but also led to increased accumulation of p-eIF2 α and CHOP (Fig. 4C and D). This was associated with increased levels of the proapoptotic BAX, BAK, BIK, and BIM (short, long, and extra long forms), as well as with increased cleavage of caspase-7 into its active form (Fig. 4D). Exposure to panobinostat also induced cell death of MCF-7 cells (Fig. 4E). Finally, we determined the effects of reducing GRP78 levels on panobinostat-mediated loss of cell survival. MCF-7 cells were transfected with shRNA to GRP78 versus the control scrambled oligonucleotides and treated with panobinostat. Partial knockdown of GRP78 sensitized breast cancer cells to cell death induced by bortezomib and low concentrations of panobinostat (10 and 20 nmol/L; Fig. 5, inset). However, knockdown of GRP78 did not affect cell death induced by higher concentrations of panobinostat (data not shown).

Discussion

The present studies show for the first time that HDAC6 functions as a deacetylase for GRP78, and inhibition of HDAC6 by the pan-HDAC inhibitor or by knockdown results in rapid acetylation of GRP78 (Fig. 6). This occurs on at least 11 lysine residues on GRP78. HDAC6 interacts with GRP78 through its second catalytic and BUZ domains, which are also the domains that are involved in the interaction of HDAC6 with hsp90 (21, 25). In a recent study, high-resolution mass spectrometry was used to show that a number of molecular chaperones are commonly acetylated on multiple lysine residues, which may affect their chaperone function during cellular stress responses (35). Our findings are consistent with this report and add GRP78 to the list of molecular chaperones that are acetylated following inhibition of HDAC6, i.e.,

HDAC6 “acetylome”, which includes a growing list of proteins (36). In transformed cells, activation of the RAS-RAF-MEK-ERK pathway and a basal state of proteotoxic stress induces GRP78 and HDAC6 levels (33, 37–39). Increased levels of GRP78 also result from an adaptive response to proteotoxic stress and UPR and confer resistance to apoptosis (2, 4–7, 20). Consistent with a previous report, our findings also show that treatment with pan-HDAC inhibitor induces UPR and GRP78 levels (31). It is noteworthy that treatment with panobinostat rapidly increases the levels of polyubiquitylated misfolded proteins, which results from panobinostat-mediated hyperacetylation of hsp90 and inhibition of its chaperone association with a number of its client proteins (22–24). Additionally, by inhibiting the role of HDAC6 in promoting the formation of protective aggregates, panobinostat treatment accentuates the induction of UPR. This mechanism has been exploited to develop the synergistic combination of panobinostat and bortezomib against multiple myeloma cells (40). However, in a previous report, TSA treatment was not observed to induce XBP1s and CHOP levels in the colon carcinoma HCT116 cells (41). This may be because, in contrast to our studies in which a more potent HDAC6 inhibitor was used, in this report, the effects of TSA were determined in a colon cancer cell type (41). This distinction is important because we show that GRP78 is a substrate for HDAC6-mediated deacetylation.

Elevated levels of GRP78 have been shown to inhibit apoptosis induced by a variety of anticancer agents (10, 18–20). Knockdown of GRP78 with siRNA was shown to induce apoptosis and sensitize glioma cells to 5-fluorouracil and CPT-11, as well as breast cancer cells to estrogen starvation (9, 18). Recently, pan-HDAC inhibitors TSA and MS-275 were shown to abrogate the

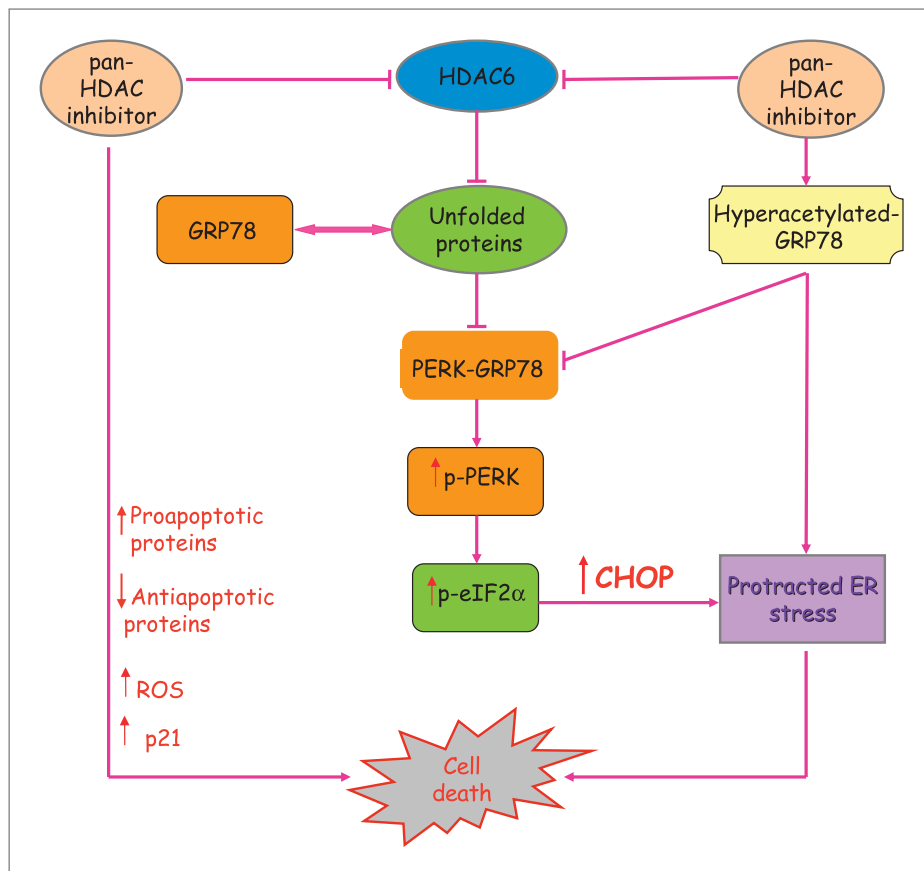


Figure 6. The mechanisms responsible for the induction of lethal UPR due to treatment with panobinostat: panobinostat-mediated inhibition of HDAC6 leads to the acetylation of GRP78, which results in the dissociation of GRP78 from the ER stress mediator PERK, leading to the activation of the p-eIF2 α -ATF4-CHOP pathway. These events lead to protracted ER stress, which results in a lethal outcome of UPR. Panobinostat induction courtesy of proapoptotic proteins, p21 and ROS, along with panobinostat-mediated downregulation of antiapoptotic proteins, are additional mechanisms of panobinostat-induced cell death.

binding of HDAC1 to GRP78 promoter and transcriptionally upregulate GRP78 (41). In this report, although ectopic overexpression of GRP78 in 293T cells was shown to confer resistance, knockdown of GRP78 sensitized MB-435 cells to TSA-induced apoptosis (41). Our findings also show that partial knockdown of GRP78 sensitized MCF-7 cells to panobinostat-induced cell death. This was observed following treatment with the relatively lower but not the higher levels of panobinostat, which may activate multiple other mechanisms of cell death (Fig. 6; refs. 42, 43). However, our findings also show that panobinostat treatment induces GRP78 acetylation, thereby neutralizing the charge in the lysine residues of GRP78 (35). This is associated with decreased binding of GRP78 with PERK and activation of lethal UPR mediated by PERK-p-eIF2 α -ATF4-CHOP signaling (Fig. 6; refs. 5, 7). Therefore, panobinostat-mediated acetylation of GRP78 may abrogate the protective effects of increased GRP78 levels, which are also induced by treatment with panobinostat. As was assessed for the individual lysine residues on hsp90, the functional significance of the panobinostat-induced acetylation of the 11 lysine residues individually remains to be determined (22).

Inhibition of hsp90 function has been shown to activate UPR by activating PERK and IRE1 α , the latter re-

sulting in the splicing of XBP1 (44). As noted above, panobinostat treatment increased Bax and Bak levels, which have been shown to activate IRE1 α signaling (6). Activated IRE1 α through its cytosolic domain has been shown to bind to the adaptor protein TNFR-associated factor 2, triggering the activation of apoptosis signal-regulating kinase 1 and the *c-Jun*-NH₂-kinase pathway, which in part may be responsible for inducing apoptosis (5, 6, 45). However, in our studies, significant *c-Jun*-NH₂-kinase activation was not observed following treatment of breast cancer cells with panobinostat (data not shown). Persistent UPR has been reported to attenuate IRE1 α signaling (7), which may be the explanation for the lack of *c-Jun*-NH₂-kinase activation observed in our studies. By contrast, we did note that panobinostat treatment triggered sustained PERK-p-eIF2 α -ATF4-CHOP signaling (Fig. 4C and D). This was associated with increased levels of BIM and BIK as well as activation of caspase-7. These perturbations have been shown to be involved in mediating a lethal UPR (7–11, 46). Taken together, panobinostat-mediated inhibition of HDAC6 and hsp90 functions, coupled with panobinostat-induced GRP78 acetylation and decreased binding to PERK (associated with the activation of the PERK-p-eIF2 α -ATF4-CHOP pathway of UPR) is most likely responsible for the lethal UPR due to panobinostat

treatment. Recent studies have shown that treatment with the pan-HDAC inhibitor vorinostat triggers autophagy in transformed cells, under those situations in which apoptosis is blocked due to inhibition of caspase activity (47). There is also accumulating evidence that ER stress could trigger autophagy in cells that do not experience lethal UPR and escape apoptosis (48, 49). Moreover, GRP78 has been shown to be necessary for ER stress-induced autophagy (50). Therefore, panobinostat-mediated GRP78 acetylation could also affect ER stress-induced autophagy in cells that escape lethal UPR. This is an important issue that remains to be comprehensively evaluated. Nevertheless, collectively our findings add to the understanding of how panobinostat treatment targets and undermines GRP78 function, thereby significantly contributing to the conditions promoting proteotoxic stress (37, 40). The findings presented here

also highlight the potential of panobinostat-based combinations that are designed to target the nononcogenic addiction of transformed cells to the proteotoxic stress in the therapy of cancer (Fig. 6; refs. 37, 40).

Disclosure of Potential Conflicts of Interest

K. Bhalla: research grant support, Novartis; Speakers' Bureau honoraria, Merck. No other potential conflicts of interest were disclosed.

Acknowledgments

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Received 10/26/2009; revised 01/19/2010; accepted 02/10/2010; published OnlineFirst 04/06/2010.

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