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Combined treatment with SAHA, bortezomib, and clarithromycin for concomitant targeting of aggresome formation and intracellular proteolytic pathways enhances ER stress-mediated cell death in breast cancer cells

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ABSTRACT

The ubiquitin–proteasome pathway and the autophagy–lysosome pathway are two major intracellular protein degradation systems. We previously reported that clarithromycin (CAM) blocks autophagy flux, and that combined treatment with CAM and proteasome inhibitor bortezomib (BZ) enhances ER-stress-mediated apoptosis in breast cancer cells, whereas treatment with CAM alone results in almost no cytotoxicity. Since HDAC6 is involved in aggresome formation, which is recognized as a cytoprotective response serving to sequester misfolded proteins and facilitate their clearance by autophagy, we further investigated the combined effect of vorinostat (suberoylanilide hydroxamic acid (SAHA)), which has a potent inhibitory effect for HDAC6, with CAM and BZ in breast cancer cell lines. SAHA exhibited some cytotoxicity along with an increased acetylation level of α-tubulin, a substrate of HDAC6. Combined treatment of SAHA, CAM, and BZ potently enhanced the apoptosis-inducing effect compared with treatment using each reagent alone or a combination of two of the three. Expression levels of ER-stress-related genes, including the pro-apoptotic transcription factor CHOP (GADD153), were maximally induced by the simultaneous combination of three reagents. Like breast cancer cell lines, a wild-type murine embryonic fibroblast (MEF) cell line exhibited enhanced cytotoxicity and maximally up-regulated Chop after combined treatment with SAHA, CAM, and BZ; however, a Chop knockout MEF cell line almost completely canceled this enhanced effect. The specific HDAC6 inhibitor tubacin also exhibited a pronounced cytocidal effect with a combination of CAM plus BZ. These data suggest that simultaneous targeting of intracellular proteolytic pathways and HDAC6 enhances ER-stress-mediated apoptosis in breast cancer cells.

1. Introduction

Endoplasmic reticulum (ER) stress is caused by an imbalance between the amount of unfolded or misfolded protein in the ER lumen and the capacity of the ER machinery to refold these proteins [1]. This stress induces a coordinated cellular response known as unfolded protein response (UPR). The main functions of UPR are to reduce the amount of protein that enters the ER by suppressing the translational rate and to increase the folding capacity of the ER via translational activation of chaperon proteins. Additionally, if proteins cannot be folded correctly in the ER, they are retrotranslocated to the cytoplasm for degradation via the ubiquitin–proteasome pathway, a process termed ER-associated degradation (ERAD), for adaptation. However, if these adaptation strategies fail, apoptosis is triggered with the induction of the pro-apoptotic transcription factor CHOP/GADD153 and with the IRE1 involved in signaling via caspase-12 [1,2]. Thus, therapeutic manipulation of this pathway using a proteasome inhibitor such as bortezomib and other reagents might interfere with the ability to deal with high protein loads and cellular stress, and this appears to induce cancer cell death.

Macroautophagy (hereafter, autophagy) is a highly conserved cellular process in eukaryotes. Intracellular proteins and organelles, including ER, are engulfed in a double-membrane vesicle known as an autophagosome, and are delivered to lysosomes for

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degradation by lysosomal hydrolases [3,4]. Autophagy has been regarded as a bulk non-selective degradation system for long-lived proteins and organelles, in contrast to the specific degradation of polyubiquitinated short-lived proteins by proteasome. However, evidence indicates a selective degradation pathway of ubiquitinated protein through autophagy via docking proteins such as p62 and the related protein NBR1, having both a microtubule-associated protein 1 light chain 3 (LC3)-interacting region and a ubiquitin-associated domain [5]. LC3 is essential for autophagy and is associated with autophagosome membranes after processing [5,6]. Binding the ubiquitinated proteins via their C-terminal ubiquitin-associated domains, p62-mediated degradation of ubiquitinated cargo occurs by selective autophagy. Thus, the two major intracellular protein degradation systems are directly linked [6,7]. In addition to the proteolytic pathways, misfolded/unfolded protein aggregates are transported and removed from the cytoplasm by dynein motors via the microtubule network to the aggresome [8]. HDAC6, a microtubule-associated deacetylase, is a component of the aggresome and has the capacity to bind both polyubiquitinated misfolded proteins and dynein motors. Therefore, HDAC6 recruits misfolded protein cargo to dynein motors for transport to aggresomes [8]. Cells deficient in HDAC6 fail to clear unfolded protein aggregates from the cytoplasm, cannot properly form aggresomes, and are hypersensitive to the accumulation of unfolded proteins [8,9]. Thus, HDAC6 is a crucial player in the cellular management of unfolded protein-induced stress. Aggresome formation is now recognized as a cytoprotective response serving to sequester unfolded proteins and facilitate their clearance by autophagy [9,10].

Evidence suggests the existence of an elaborate intracellular network system for processing unfolded proteins. Indeed, we have reported that the macrolide antibiotic clarithromycin (CAM) blocks autophagy flux and that combined treatment with CAM and proteasome inhibitor bortezomib (BZ) enhanced ER-stress-mediated apoptosis by blocking two major intracellular protein degradation systems in breast cancer and myeloma cells; however, treatment with CAM alone resulted in almost no cytotoxicity [11,12]. Therefore, simultaneously targeting aggresome formation and intracellular proteolytic pathways appears to load ER-stress further. In the present study, we attempted to use vorinostat (suberoylanilide hydroxamic acid (SAHA)), which is an orally bioavailable HDAC inhibitor approved by the Food and Drug Administration for treatment of cutaneous T-cell lymphoma, to block aggresome formation [13]. SAHA has a potent inhibitory effect against HDAC6 with IC_{50} of 37 nM [14]. A phase I-II study on metastatic breast cancer reported that SAHA was safely combined with paclitaxel and bevacizumab using an intermittent schedule along with the induction of hyperacetylation of histone and non-histone proteins (e.g., α-tubulin and Hsp90) in vivo [15]. We demonstrate here that combined treatment with SAHA, CAM, and BZ exhibited pronounced ER-stress-mediated breast cancer cell death, providing a novel strategy of ER-stress loading therapy for refractory-metastatic breast cancer patients.

2. Materials and methods

2.1. Reagents

SAHA was purchased from Cayman Chemical Company (Ann Arbor, MI), and tubacin [16] was purchased from Sigma–Aldrich (St. Louis, MO). These reagents were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM as a stock solution. BZ was purchased from Toronto Research Chemical, Inc. (North York, Ontario, Canada) and dissolved in DMSO at a concentration of 1 mM as a stock solution. CAM, purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), was dissolved in 95% ethanol at a concentration of 5 mg/ml as a stock solution.

2.2. Cell lines and culture conditions

For this study, breast cancer cell lines (e.g., MDA-MB-231 and BT474 cells) were kind gifts from Dr. Keiichi Iwaya (Department of Basic Pathology, National Defense Medical College, Saitama), and MDA-MB-468 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A $CHOP^{-/-}$MEF cell line (CHOP-KO-DR) established from a 13.5-day-old $CHOP^{-/-}$ mouse embryo by SV-40 immortalization and a $CHOP^{+/+}$MEF cell line

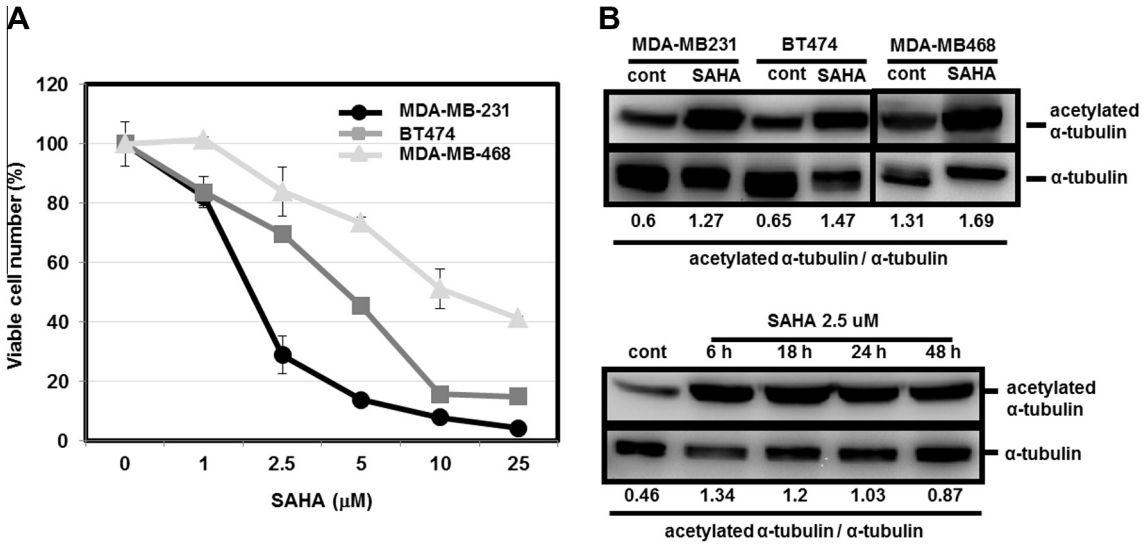

Fig. 1. Cell-growth inhibition along with acetylation of α-tubulin after treatment with SAHA in breast cancer cell lines. (A) MDA-MB-231, BT474, and MDA-MB-468 cells were treated with SAHA at various concentrations for 72 h. The number of viable cells was assessed by CellTiter Blue as described in Section 2. (B) Upper panel: after treatment with 2.5 μM of SAHA (in MDA-MB-231 and MDA-MB-468 cells for 48 h, in BT474 cells for 72 h), cells were lysed, separated by 11.25% SDS–PAGE, and immunoblotted with anti-acetylated α-tubulin mAb as well as anti-α-tubulin. Lower panel: MDA-MB-231 cells were treated with 2.5 μM of SAHA for 6–48 h. Cells were then lysed, separated by 11.25% SDS–PAGE, and immunoblotted with anti-acetylated α-tubulin mAb as well as anti-α-tubulin. Each number indicates the expression ratio of acetylated α-tubulin to whole α-tubulin, as assessed by densitometry.

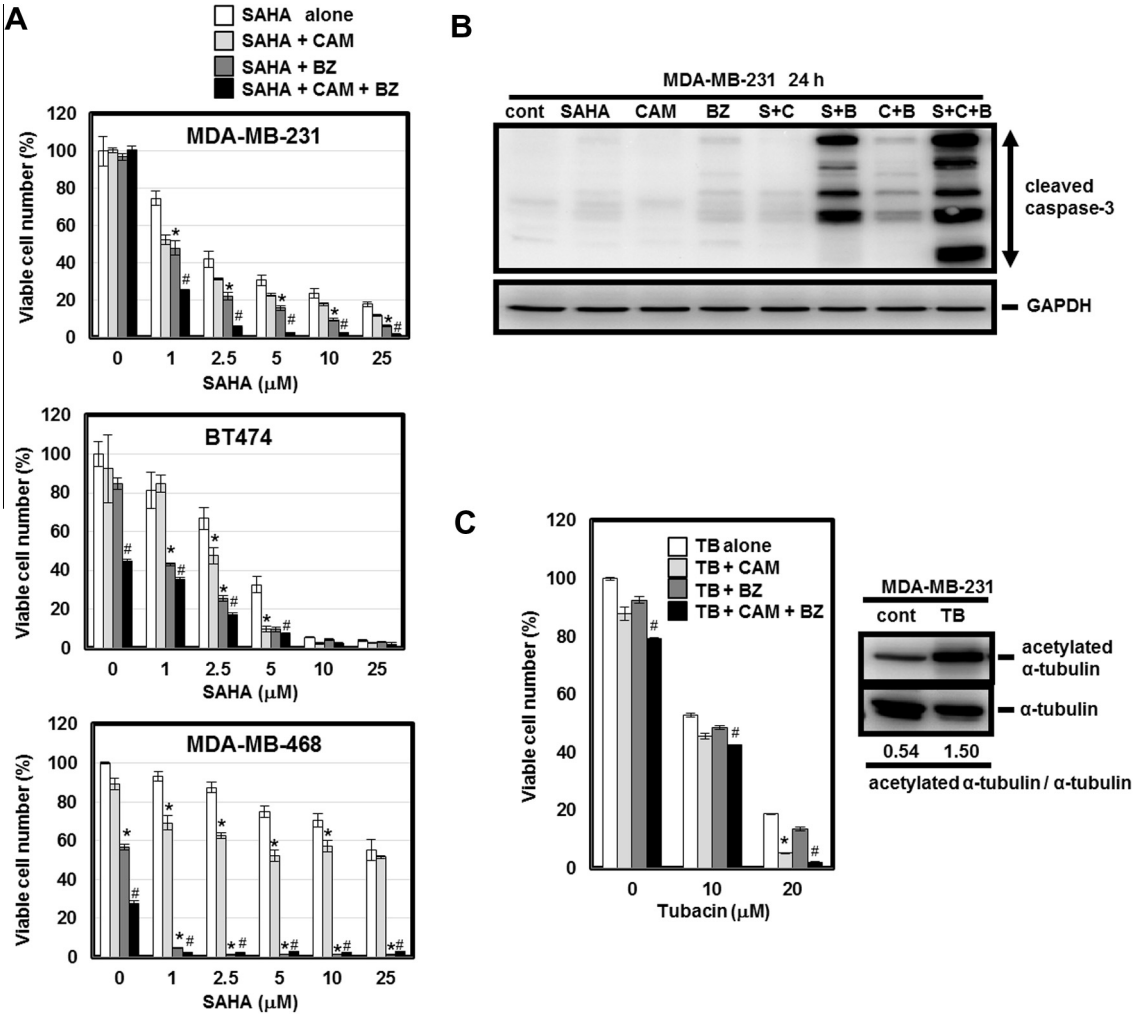

Fig. 2. Enhanced cell-growth inhibition and apoptosis induction by combined treatment with SAHA or a specific inhibitor of HDAC6, tubacin, to CAM plus BZ in breast cancer cell lines. (A) MDA-MB-231 and MDA-MB-468 cells were treated with SAHA at various concentrations in the presence of BZ (15 nM) and CAM (50 μg/ml) for 48 h. Since BT474 cells were less sensitive to BZ, the culture conditions were 25 nM of BZ and 50 μg/ml of CAM for 96 h. Numbers of viable cell were assessed by CellTiter Blue. $^{*}p < 0.05$ SAHA alone vs. SAHA + CAM or SAHA + BZ. $^{\#}p < 0.05$ SAHA + CAM + BZ vs. SAHA + CAM/SAHA + BZ. (B) MDA-MB-231 cells were treated with SAHA (2.5 μM) and/or CAM (50 μg/ml) and/or BZ (15 nM) for 24 h. Cellular proteins were lysed, separated by 15% SDS–PAGE, and immunoblotted with anti-cleaved caspase-3 Ab. Immunoblotting with anti-GAPDH mAb was performed as an internal control. S: SAHA, C: CAM, B: BZ. (C) MDA-MB-231 cells were cultured with tubacin (TB) at various concentrations for 48 h in the presence or absence of 50 μg/ml of CAM with/without 15 nM of BZ. The number of viable cells was assessed as described in Section 2. $^{*}p < 0.05$ TB alone vs. TB + CAM. $^{\#}p < 0.05$ TB + CAM + BZ vs. TB + CAM/TB + BZ.

(DR-wild-type) established by SV-40 immortalization as a control cell line for CHOP-KO-DR were obtained from ATCC. Precise culture conditions were previously described elsewhere [12].

2.3. Assessment of the number of viable cells among cultured cells

The number of viable cells was assessed by CellTiter Blue, a cell viability assay kit (Promega Co., Madison, WI), with fluorescence measurements of 570 nm for excitation and 590 nm for fluorescence emission.

2.4. Immunoblotting

Immunoblotting was performed as previously described [11]. The transferred membranes were probed with first antibodies (Abs). Regarding first Abs, anti-HDAC6 Ab, anti-α-tubulin monoclonal (m) Ab, anti-acetylated α-tubulin mAb, anti-p62 mAb, and anti-GAPDH mAb were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-CHOP mAb and anti-cleaved caspase-3 Ab (Asp175) were obtained from Cell Signaling Technology (Danvers, MA), and anti-LC3B Ab was obtained from Novus Biologicals, Inc. (Littleton, CO). Immunoreactive proteins were detected with horseradish peroxidase-conjugated second Abs and an enhanced chemiluminescence reagent (ECL) (Millipore, Bedford, MA). Densitometry was performed using a Molecular Imager, ChemiDoc XRS System (Bio-Rad, Richmond, CA).

2.5. Gene expression analysis

Real-time PCR was performed to assess gene expression, as we previously described in detail [12].

2.6. Transfection of CHOP siRNA

For the gene silence of *CHOP* in MDA-MB-231 cells, CHOP siRNA and control siRNA, purchased from Invitrogen (Paisley, UK) and whose sequences are described below, were diluted to a final concentration of 33 nM in Opti-Mem I (Invitrogen). Transfection was performed with the cells at 50% confluency, using Lipofectamine

RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions:

CHOP sense: UUUCCUGCUUGAGCCGUUCAUUCUC.
CHOP antisense: GAGAAUGAACGGCUCAAGCAGGAAA.

3. Results and discussion

3.1. Combination of SAHA, CAM, and BZ enhances cytotoxicity and apoptosis induction in breast cancer cell lines

SAHA induced cell growth inhibition in a dose-dependent manner in all three breast cancer cell lines tested. However, cytotoxic sensitivity to SAHA varied among the cell lines tested: IC_{50} (50% inhibitory concentrations) was 1.91 μM for MDA-MB-231, 4.55 μM for BT474, and 12.6 μM for MDA-MB-468 (Fig. 1A). As our previous report indicated, proteasome inhibitor BZ induced cell growth inhibition in a dose-dependent manner in MDA-MB-231 cells (IC_{50} 39.3 nM) and MDA-MB-468 cells (IC_{50} 21.2 nM). BT474 cells exhibited less sensitivity to BZ (Supplementary Fig. S1) [11]. Although MDA-MB-468 cells exhibited the most potent cell growth inhibition in response to BZ, they were the least sensitive to SAHA. This result suggests diversity in cell-line sensitivity to SAHA and BZ. Treatment with CAM resulted in almost no cytotoxicity in all these cell lines at a concentration of less than 50 μg/ml (Supplementary Fig. S2).

It has been reported that SAHA potently inhibits HDAC6 (IC_{50} 37 nM) as well as HDAC1 (IC_{50} 30 nM) and HDAC2 (IC_{50} 49 nM), but not HDAC4 (IC_{50} > 10,000 nM) [14,15]. HDAC6 can bind to polyubiquitinated misfolded proteins by its zinc finger domain as well as deacetylates α-tubulin. These functions recruit misfolded protein cargo to dynein motors and transport it to the microtubule organizing center (MTOC) for aggresome formation [8].

As indicated in Fig. 1B, treatment with SAHA increased the acetylated status of α-tubulin within 6 h treatment, and this effect persisted for at least 48 h. Although BT474 cells are less sensitive to SAHA in cell growth inhibition, an increased acetylation level of α-tubulin was detected after 2.5 μM of SAHA treatment.

We and others have reported that proteasome inhibitor BZ induces autophagy [17,18]. CAM and azithromycin inhibit the later part of the autophagy process and as a result block autophagy flux and accumulation of autophagosomes in cytoplasm [19,20]. In addition, concomitant inhibition of the ubiquitin–proteasome system by BZ and the autophagy–lysosome system by CAM resulted in induction of ER-stress-mediated cell death and increased accumulation of the intracellular ubiquitin-conjugated proteins in myeloma cells [12]. Since aggresome formation sequesters unfolded proteins and facilitates their clearance by autophagy in part [9,10], we speculated that simultaneously inhibiting intracellular protein degradation and aggresome formation further potentiates ER-stress-mediated cell death. As we expected, the combination of SAHA, CAM, and BZ resulted in prominent enhancement of cell-growth inhibition in all breast cancer cell lines (Fig. 2A). It was noteworthy that although MDA-MB-468 cells were less sensitive to SAHA, the combination of SAHA with either BZ or BZ and

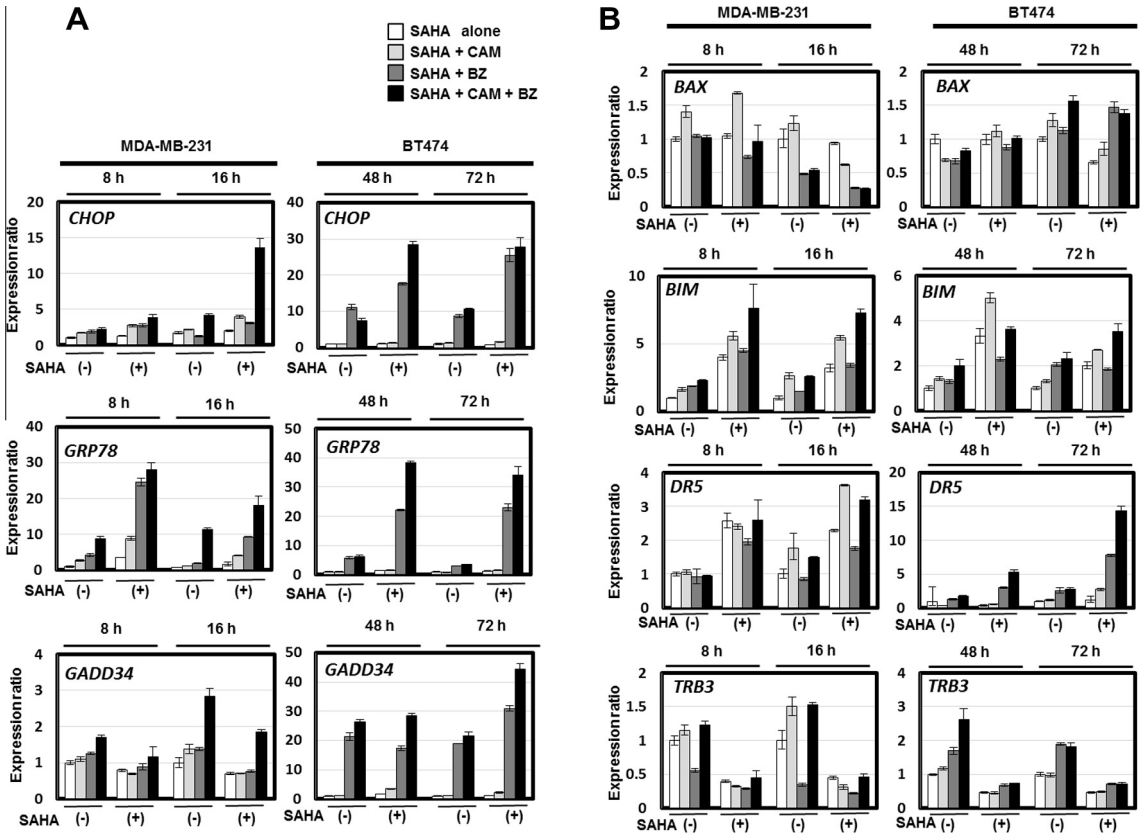

Fig. 3. Expression profiles of ER-stress-related genes and *CHOP*-regulated genes after treatment with SAHA in the presence or absence of CAM with/without BZ in breast cancer cell lines. After treatment with/without SAHA (2.5 μM) ± CAM (50 μg/ml) ± BZ (15 nM in MDA-MB-231,25 nM in BT474) for 8 and 16 h in MDA-MB-231 cells and 48 and 72 h in BT474 cells, the expression levels of ER-stress-related genes (A) such as *CHOP*, *GRP78*, and *GADD34*, and pro-apoptotic genes (B) such as *BAX*, *BIM*, *DR5*, and *TRB3*, which are all transcriptionally regulated by *CHOP*, were all analyzed by real-time PCR. The data of the real-time PCR products for each gene were standardized to *GAPDH* as an internal control. The expression levels were compared with those in untreated cells.

CAM resulted in pronounced cell-growth inhibition. To investigate whether enhanced cytotoxicity is mediated through apoptosis, we next performed flow cytometry. Combined treatment using BZ plus SAHA enhanced both the annexin V-positive and the propidium iodide (PI)–positive MDA-MB-231 cell population, compared with SAHA or BZ alone (Supplementary Fig. S3). Although treatment with CAM alone had almost no effect, the addition of CAM to BZ and SAHA containing culture medium resulted in pronounced induction of annexin V-positive/PI-positive cells. Immunoblotting with anti-cleaved caspase-3 indicated enhanced activation of caspase-3 by combined treatment with three reagents in MDA-MB-231 cells (Fig. 2B). Not shown is that morphological features of MDA-MB-231 cells exhibited fragmented DNA and apoptotic bodies characteristic of cells undergoing apoptosis under these conditions. All these data indicate enhanced apoptosis induction by combined treatment with three reagents. In addition, the combination of a specific inhibitor of HDAC6, tubacin [21] plus CAM and BZ, resulted in enhanced cytotoxicity in MDA-MB-231 cells (Fig. 2C). This result confirmed that inhibition of HDAC6 was involved in pronounced apoptosis induction in the presence of CAM and BZ.

3.2. Combined treatment with SAHA, CAM, and BZ results in further loading of ER-stress in breast cancer cells

We next examined the induction of ER-stress-related genes such as *CHOP*, *GRP78*, and *GADD34* in MDA-MB-231 and BT474 cells. Real-time PCR indicated that SAHA plus BZ is a substantial combination for up-regulation of all ER-stress-related genes. However, the addition of CAM to BZ and SAHA resulted in further induction of ER-stress genes, as well as enhanced apoptosis induction (Fig. 3A). The kinetics of gene induction differed between MDA-MB-231 and BT474 cell lines; expression profiles of MDA-MB-231 cells changed within 16 h exposure, but required more than 48 h in BT474 cells. It was noteworthy that longer exposure time to these reagents was required to exhibit cytotoxicity in BT474 cells than in MDA-MB-231 cells (Fig. 2A). BT474 cells grow much more slowly than other cell lines; thus, cellular metabolism, including protein synthesis, in these cells may be slower than in MDA-MB-231 cells. Indeed, in myeloma cells, it was reported that Myc expression directly correlated with protein synthesis rates, percentage of aggresome-positive cells, sensitivity to BZ, and SAHA-induced cell death [22]. Therefore, these data support the linkage between cytotoxicity and ER-stress loading, as indicated in our previous reports [11,12]. Expression profiles of ER-stress-related genes such as *CHOP*, *GRP78*, and *GADD34* demonstrated that treatment with SAHA plus CAM plus BZ was the most potent combination for ER-stress loading, compared with each reagent alone or a combination of two of these three reagents (Fig. 3A). Treatment with CAM alone had little effect on ER-stress-related gene induction, whereas the addition of either BZ or SAHA had a substantial effect on *GRP 78* inductions. Furthermore, the simultaneous combination of these three reagents resulted in further enhancement of ER-stress-related gene induction (Fig. 3A). CHOP

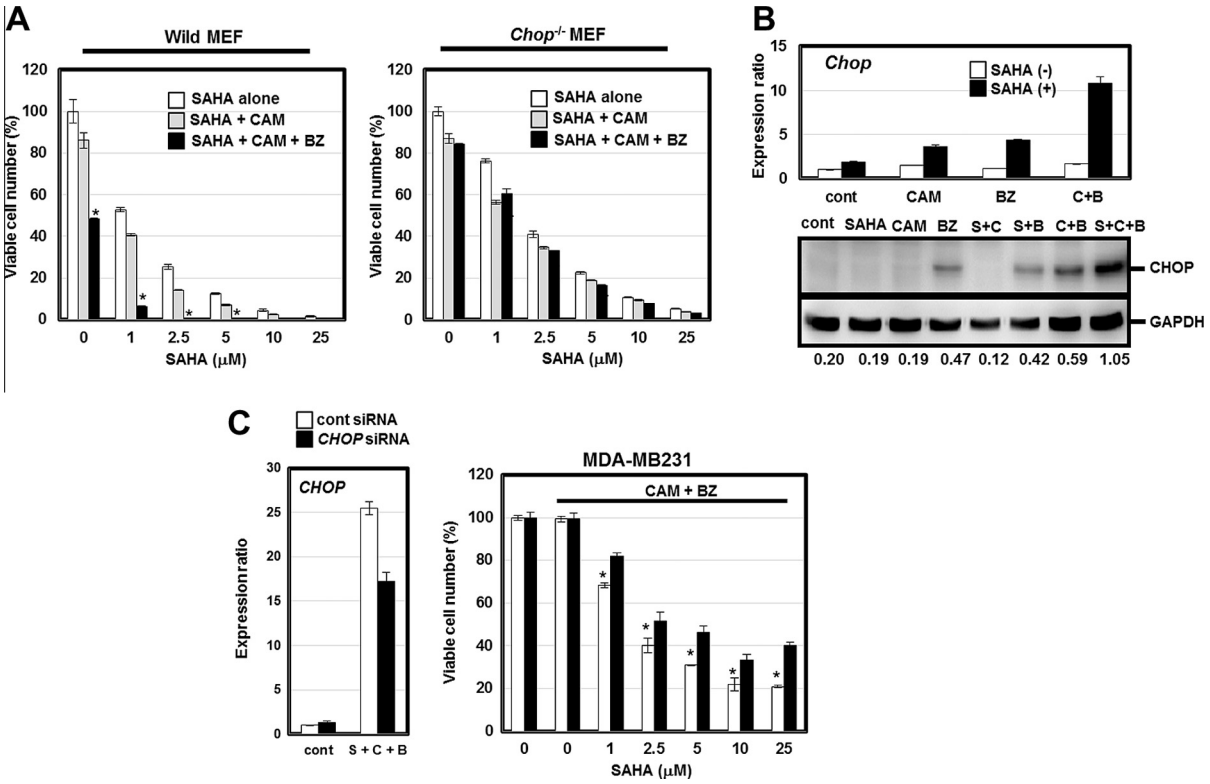

Fig. 4. Involvement of CHOP induction in pronounced cytotoxicity by combined treatment with SAHA, CAM, and BZ. (A) Cell growth inhibition after combined treatment with SAHA, CAM (25 μg/ml), and BZ (5 nM) for 48 h in a wild-type MEF cell line and a $CHOP^{-/-}$ MEF cell line. $^{*}p < 0.05$ SAHA + CAM + BZ vs. SAHA + CAM. (B) *Chop* induction after treatment with SAHA (2.5 μM) ± CAM (25 μg/ml) ± BZ (5 nM) for 24 h in a wild MEF cell line. Upper panel: the expression levels of *Chop* were analyzed by real-time PCR, and the data of the real-time PCR products were standardized to *Gapdh* as an internal control. The expression levels were compared with those in untreated control cells. Lower panel: cellular proteins were separated by 11.25% SDS–PAGE and immunoblotted with anti-CHOP mAb. Immunoblotting with anti-GAPDH mAb was performed as an internal control. (C) Effect of CHOP knockdown by siRNA in MDA-MB-231 cell-growth inhibition after combined treatment with SAHA, CAM, and BZ. Left panel: after knockdown with CHOP siRNA, cells were subsequently treated with SAHA (2.5 μM) + CAM (50 μg/ml) + BZ (15 nM) for 48 h. The expression levels of *CHOP* were then analyzed by real-time PCR. The data of the real-time PCR products were standardized to *GAPDH* as an internal control. Right panel: after knockdown with CHOP siRNA, MDA-MB-231 cells were subsequently treated with various concentrations of SAHA in the presence of CAM (50 μg/ml) and BZ (15 nM) for 72 h. The number of viable cells was assessed by CellTiter Blue as described in Section 2. $^{*}p < 0.05$ *CHOP* siRNA vs. cont siRNA, S: SAHA, C: CAM, B: BZ.

is thought to be the critical mediator of ER-stress-induced apoptosis, and studies using *Chop*-null mice have established the role of CHOP in ER-stress-induced apoptosis in a number of disease models [23–26]. CHOP is present in the cytosol under non-stressed conditions, and ER stress leads to its induction and nuclear accumulation [26]. Expression of CHOP is regulated mainly at the transcriptional level [23]. However, CHOP protein undergoes phosphorylation by the p38 MAP kinase family, which enhances its transcriptional ability for various pro-apoptotic genes, including *BIM*, *BAX*, *TRB3*, and *DR5* [1,26].

As indicated in Fig. 3B, all pro-apoptotic genes transcriptionally regulated by CHOP appeared to be pronounced with combined treatment. In particular, *BIM* gene coding BH3-only protein BIM exhibited prominent up-regulation in MDA-MB-231 cells within 8 h; however, in BT474 cells, *DR5* gene coding death receptor 5, also known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2, demonstrated pronounced induction after 72 h exposure to SAHA plus CAM plus BZ. Although the gene expression profiles of the downstream CHOP exhibited some variation, these data strongly suggest that combined treatment with three reagents results in pronounced ER-stress loading followed by induction and activation of pro-apoptotic transcription factor CHOP and successive up-regulation of various pro-apoptotic proteins, such as BIM and DR5.

3.3. CHOP is involved in enhanced cytotoxicity by combined treatment with SAHA, CAM, and BZ

To prove that ER-stress-mediated CHOP induction is involved in pronounced cytotoxicity, we used a *Chop* knockout murine embryonic fibroblast (MEF) cell line (Fig. 4A). Pronounced cytotoxicity was observed with combined treatment with SAHA, CAM, and BZ in a wild-type MEF cell line, as well as in breast cancer cell lines. Both real-time PCR and immunoblotting with anti-CHOP mAb indicated maximum up-regulation by combined treatment with three reagents (Fig. 4B). Notably, this enhanced cytotoxicity was almost completely canceled in the *Chop* knockout MEF cell line (Fig. 4A). In addition, knockdown of CHOP by siRNA attenuated enhanced cytotoxicity in MDA-MB-231 cells (Fig. 4C). All these data support the mediation of enhanced cytotoxicity through CHOP induction. However, some cytotoxicity was still observed in response to SAHA in the *Chop* knockout MEF cell line (Fig. 4A). Since SAHA is a pan-HDAC inhibitor, other epigenetic changes may be involved in cytotoxicity [27]. In addition to its central role in eliminating protein aggregation, HDAC6 has been reported to be involved in mitochondrial transport and oxidative stress [28,29]. Therefore, the cytotoxic effect of SAHA may not be simply converged to CHOP induction.

All data indicates that combined treatment with SAHA, CAM, and BZ had a pronounced apoptosis-inducing effect on breast cancer cell lines. This enhanced effect was at least in part due to ER-stress-mediated CHOP induction. This result provides novel insight into ER-stress loading therapy by concomitant targeting of aggresome formation and intracellular proteolytic pathways for refractory-metastatic breast cancer patients.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.032.

References

[1] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat. Rev. Mol. Cell Biol. 8 (2007) 519–529.
[2] T. Verfaillie, M. Salazar, G. Velasco, P. Agostinis, Linking ER stress to autophagy: potential implications for cancer therapy, Int. J. Cell Biol. (2010) 930509.
[3] N. Mizushima, B. Levine, Autophagy in mammalian development and differentiation, Nat. Cell Biol. 12 (2010) 823–830.
[4] F. Janku, D.J. McConkey, D.S. Hong, R. Kurzrock, Autophagy as a target for anticancer therapy, Nat. Rev. Clin. Oncol. 8 (2011) 528–539.
[5] V. Kirkin, D.G. McEwan, I. Novak, I. Dikic, A role for ubiquitin in selective autophagy, Mol. Cell 34 (2009) 259–269.
[6] V.I. Korolchuk, F.M. Menzies, D.C. Rubinsztein, Mechanisms of cross-talk between the ubiquitin–proteasome and autophagy–lysosome systems, FEBS Lett. 584 (2010) 1393–1398.
[7] I. Tabas, D. Ron, Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress, Nat. Cell Biol. 13 (2011) 184–190.
[8] Y. Kawaguchi, J.J. Kovacs, A. McLaurin, J.M. Vance, A. Ito, T.P. Yao, The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress, Cell 115 (2003) 727–738.
[9] A. Rodriguez-Gonzalez, T. Lin, A.K. Ikeda, T. Simms-Waldrip, C. Fu, K.M. Sakamoto, Role of the aggresome pathway in cancer: targeting histone deacetylase 6-dependent protein degradation, Cancer Res. 68 (2008) 2557–2560.
[10] T. Simms-Waldrip, A. Rodriguez-Gonzalez, T. Lin, A.K. Ikeda, C. Fu, K.M. Sakamoto, The aggresome pathway as a target for therapy in hematologic malignancies, Mol. Genet. Metab. 94 (2008) 283–286.
[11] S. Komatsu, K. Miyazawa, S. Moriya, A. Takase, M. Naito, M. Inazu, N. Kohno, M. Itoh, A. Tomoda, Clarithromycin enhances bortezomib-induced cytotoxicity via endoplasmic reticulum stress-mediated CHOP (GADD153) induction and autophagy in breast cancer cells, Int. J. Oncol. 40 (2012) 1029–1039.
[12] S. Moriya, X.F. Che, S. Komatsu, A. Abe, T. Kawaguchi, A. Gotoh, M. Inazu, A. Tomoda, K. Miyazawa, Macrolide antibiotics block autophagy flux and sensitize to bortezomib via endoplasmic reticulum stress-mediated CHOP induction in myeloma cells, Int. J. Oncol. 42 (2013) 1541–1550.
[13] E.A. Olsen, Y.H. Kim, T.M. Kuzel, T.R. Pacheco, F.M. Foss, S. Parker, S.R. Frankel, C. Chen, J.L. Ricker, J.M. Arduino, M. Duvic, Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma, J. Clin. Oncol. 25 (2007) 3109–3115.
[14] M. Namdar, G. Perez, L. Ngo, P.A. Marks, Selective inhibition of histone deacetylase 6 (HDAC6) induces DNA damage and sensitizes transformed cells to anticancer agents, Proc. Natl. Acad. Sci. USA 107 (2010) 20003–20008.
[15] B. Ramaswamy, W. Fiskus, B. Cohen, C. Pellegrino, D.L. Hershman, E. Chuang, T. Luu, G. Somlo, M. Goetz, R. Swaby, C.L. Shapiro, V. Stearns, P. Christos, I. Espinoza-Delgado, K. Bhalla, J.A. Sparano, Phase I-II study of vorinostat plus paclitaxel and bevacizumab in metastatic breast cancer: evidence for vorinostat-induced tubulin acetylation and Hsp90 inhibition in vivo, Breast Cancer Res. Treat. 132 (2012) 1063–1072.
[16] G.I. Aldana-Masangkay, A. Rodriguez-Gonzalez, T. Lin, A.K. Ikeda, Y.T. Hsieh, Y.M. Kim, B. Lomenick, K. Okemoto, E.M. Landaw, D. Wang, R. Mazitschek, J.E. Bradne, K.M. Sakamoto, Tubacin suppresses proliferation and induces apoptosis of acute lymphoblastic leukemia cells, Leuk. Lymphoma 52 (2011) 1544–1555.
[17] T. Kawaguchi, K. Miyazawa, S. Moriya, T. Ohtomo, X.F. Che, M. Naito, M. Itoh, A. Tomoda, Combined treatment with bortezomib plus bafilomycin A1 enhances the cytocidal effect and induces endoplasmic reticulum stress in U266 myeloma cells: crosstalk among proteasome, autophagy–lysosome and ER stress, Int. J. Oncol. 38 (2011) 643–654.
[18] B. Hoang, A. Benavides, Y. Shi, P. Frost, A. Lichtenstein, Effect of autophagy on multiple myeloma cell viability, Mol. Cancer Ther. 8 (2009) 1974–1984.
[19] M. Nakamura, Y. Kikukawa, M. Takeya, H. Mitsuya, H. Hata, Clarithromycin attenuates autophagy in myeloma cells, Int. J. Oncol. 37 (2010) 815–820.
[20] M. Renna, C. Schaffner, K. Brown, Azithromycin blocks autophagy and may predispose cystic fibrosis patients to mycobacterial infection, J. Clin. Invest. 121 (2011) 3554–3563.
[21] S.J. Haggarty, K.M. Koeller, J.C. Wong, C.M. Grozinge, S.L. Schreiber, Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation, Proc. Natl. Acad. Sci. USA 100 (2003) 4389–4394.
[22] S.T. Nawrocki, J.S. Carew, K.H. Maclean, J.F. Courage, P. Huang, J.A. Houghton, J.L. Cleveland, F.J. Giles, D.J. McConkey, Myc regulates aggresome formation, the induction of Noxa, and apoptosis in response to the combination of bortezomib and SAHA, Blood 112 (2008) 2917–2926.
[23] H. Zinszner, M.M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum, Genes Dev. 12 (1998) 982–995.
[24] S. Oyadomari, A. Koizumi, K. Takeda, T. Gotoh, S. Akira, E. Arak, M. Mori, Targeted disruption of the chop gene delays endoplasmic reticulum stress-mediated diabetes, J. Clin. Invest. 109 (2002) 525–532.

[25] R.M. Silva, V. Ries, T.F. Oo, O. Yarygina, V. Jackson-Lewis, CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism, J. Neurochem. 95 (2005) 974–986.

[26] D. Ron, J.F. Habener, CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription, Genes Dev. 6 (1992) 439–453.

[27] G. Giannini, W. Cabri, C. Fattorusso, M. Rodriquez, Histone deacetylase inhibitors in the treatment of cancer: overview and perspectives, Future Med. Chem. 4 (2012) 1439–1460.

[28] J. Haakenson, X. Zhang, HDAC6 and ovarian cancer, Int. J. Mol. Sci. 14 (2013) 9514–9535.

[29] G. Li, H. Jiang, M. Chang, H. Xie, L. Hu, HDAC6 α-tubulin deacetylase: a potential therapeutic target in neurodegenerative diseases, J. Neurol. Sci. 304 (2011) 1–8.