Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells

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Abstract:
Histone deacetylase (HDAC) inhibitors (HDIs) induce endoplasmic reticulum (ER) stress and apoptosis, while promoting autophagy, which promotes cancer cell survival when apoptosis is compromised. Here, we determined the in vitro and in vivo activity of the combination of the pan-HDI panobinostat (PS) and the autophagy inhibitor chloroquine (CL) against human estrogen/progesterone receptor and HER2 (triple)-negative breast cancer (TNBC) cells. Treatment of MB-231 and SUM159PT cells with PS disrupted the hsp90/histone deacetylase 6/HSF1/p97 complex, resulting in the upregulation of heat shock proteins. This was accompanied by the induction of enhanced autophagic flux as evidence by increased expression of LC3B-II and the degradation of the autophagic substrate p62. Treatment with PS also induced the accumulation and co-localization of p62 with LC3B-II in cytosolic foci as evidenced by immunofluorescent confocal microscopy. Inhibition of PS-induced autophagic flux by CL markedly induced the accumulation of polyubiquitylated proteins and p62, caused synergistic cell death of MB-231 and SUM159PT cells and inhibited mammosphere formation in MB-231 cells, compared to treatment with each agent alone. Finally, in mouse mammary fat pad xenografts of MB-231 cells, a tumor size-dependent induction of heat shock response, ER stress and autophagy was observed. Co-treatment with PS and CL resulted in the reduced tumor burden and increased the survival of MB-231 breast cancer xenografts. Collectively, our findings demonstrate that co-treatment with an autophagy inhibitor and pan-HDI e.g., CL and PS results in accumulation of toxic polyubiquitylated proteins, exerts superior inhibitory effects on TNBC cell growth and increases the survival of TNBC xenografts.
**Introduction:**

The “stress phenotype”, a hallmark of cancers, is collectively induced by hypoxia, DNA damage, reduced supply of nutrients, accumulated misfolded proteins, acidosis and increased reactive oxygen species (1,2). Aneuploidy and the resulting dosage imbalance of genes and proteins, leads to protein-misfolding/denaturing (proteotoxic) stress in cancer cells (1,2). The accumulated misfolded proteins disrupt the multi-protein complex consisting of hsp90, HSF1, HDAC6, and p97 (3,4). This releases and activates HSF1, which induces the heat shock proteins (hsp), including hsp40, hsp70 and hsp90 (5,6). The heat shock proteins promote proper protein folding, but also inhibit both the intrinsic and extrinsic pathways of apoptosis (5-7). Other adaptive responses to remove misfolded proteins from cells include the induction of aggresome formation and autophagy (8-10). Although it occurs at low basal levels for homeostatic functions involving protein and organelle turnover, autophagy is up-regulated under conditions where there is increased need for intracellular nutrients and energy. This is the case during periods of starvation and growth factor withdrawal, or when there is need to eliminate intracellular components generated by various forms of cellular stress, including oxidative stress, hypoxia, toxic protein aggregates and therapeutic stresses (9-12). Treatment of cancer cells with pan-HDI, such as vorinostat and PS, is known to induce autophagy in cancer cells, especially if caspase activity or apoptosis induction is inhibited (13). Accumulation of misfolded proteins in the cytosol also induces the HDAC6-dependent shuttling of misfolded proteins along microtubules into protective, perinuclear aggregates called aggresomes, which are further cleared by autophagy (8,9). Several studies have shown that by inhibiting HDAC6, treatment with vorinostat or PS inhibits aggresome formation and induces ER stress response represented by upregulation of the chaperone GRP78 and activation of the transcription factors ATF4 and CHOP (15-17). ER stress response and activation of ATF4 and CHOP has also been shown to induce autophagy genes, thereby linking ER stress to autophagy (18).

There are more than 30 autophagy related (Atg) genes in yeast, which encode proteins that are essential for the execution of autophagy (19). The initiation of the phagophore formation is linked to the activation of the lipid kinase PI3KC3 complex, which consists of Beclin 1 (Atg6), Atg14, Vps (vacuolar protein sorting) 34 and Vps15 (11,20). The phagophore expansion and completion of autophagosome formation is mediated by the two ubiquitin-like Atg12-Atg5 and LC3-phosphatidylethanolamine (PE) conjugation pathways (11,20). The fusion of autophagosomes with lysosomes results in degradation of autophagosomal protein aggregates, damaged organelles and macromolecules, leading to the removal of
toxic protein aggregates and recycling of metabolites into the cytosol (11). During autophagy, a cytosolic form of LC3B (LC3B-I) is conjugated to PE to form the LC3-PE conjugate (LC3B-II), which is recruited to autophagosomal membranes (11,20). LC3B-II is degraded by lysosomal hydrolases after the fusion of autophagosomes with lysosomes. (11,20). The ubiquitin- and LC3B-II-binding autophagy adaptor p62/sequestome1 transports polyubiquitylated misfolded and aggregated proteins, as well as damaged organelles, for degradation through autophagy (21). Following fusion of the autophagosome to lysosome, p62 and polyubiquitylated proteins are degraded (22). When autophagy is inhibited, there is accumulation of p62 and polyubiquitylated proteins (22).

Autophagy has now been recognized as an important tumor suppressor mechanism due to its role in preventing oxidative stress and genomic instability and in the clearance of p62 (22,23). Autophagy-deficient mice due to Atg7 loss develop liver tumors and accumulate p62 (24,25). However, autophagy can also allow tumor cells to survive stress due to hypoxia, nutrient withdrawal and cancer therapeutic agents (11,12). Based on the accumulating evidence for the role of autophagy in promoting tumor survival under stressful conditions, the strategy of abrogating autophagy with chloroquine (CL) (and hydroxychloroquine) for promoting tumor regression has been recently tested in pre-clinical and clinical settings (12,14). CL is a lysomotropic agent that inhibits autophagic flux by disturbing lysosome pH and function (12). Since treatment with pan-HDI is known to induce autophagy, in the present study, we investigated the molecular mechanism(s) of cell death following co-treatment with PS and CL in human triple-negative breast cancer (TNBC) cells that lack expression of estrogen receptor, progesterone receptor and HER2 (26). TNBC cells often express genes characteristic of the basal epithelial and normal breast myoepithelial cells (27). TNBCs are associated with high rates of relapse following chemotherapy (28,29) Therefore, it is important to develop novel and effective therapies targeting TNBCs (28,29). Our studies demonstrate that, in vivo, advanced TNBC displays evidence for ER stress and autophagy. We also show that treatment of TNBC cells with PS accentuates ER stress and autophagy, and co-treatment with CL significantly enhances the in vitro and in vivo efficacy of PS against TNBC cells.

Materials and Methods:
Reagents and Antibodies: Panobinostat (PS) was provided by Novartis Pharmaceuticals Inc. Chloroquine (CL) was obtained from Sigma-Aldrich. The chemical structures for PS and CL are
provided in Supplemental Figure 1. Anti-phosho-eIF2α, eIF2α, HSF1, Beclin 1, LC3B-II and ATG7 antibodies were purchased from Cell Signaling Technology. Anti-HDAC6, GRP78 and CHOP antibodies were purchased from Santa Cruz Biotechnology, Inc., Monoclonal anti-hsp90 antibodies were obtained from Assay Designs. Anti-Beclin 1, p97 and p62 antibodies were purchased from BD Transduction Laboratories and anti-ubiquitin antibodies were obtained from Covance. Anti-Ac K69/hsp90 antibodies were raised against acetylated lysine 69 of hsp90 as described previously (30). Anti-Vps34 antibodies were purchased from Echelon Biosciences, Inc.

**Cell culture:** MDA-MB-231 cells were obtained from American Type Culture Collection (HTB-26). SUM159PT cells were obtained from University of Michigan Human Breast Cancer Cell/Tissue Bank/Asterand (Ann Arbor, MI). Both cell lines were banked after receipt, and passaged for less than 6 months before use in this study. The American Type Culture Collection and Asterand characterize cell lines using short tandem repeat polymorphism analysis. MDA-MB-231 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. SUM159PT cells were cultured in Ham’s-F12 medium containing 5% FBS, 1% Penicillin-Streptomycin, 5 µg/ml insulin and 1 µg/ml hydrocortisone. 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) and pelleted prior to the performance of the studies described below.

**Western Blot Analyses and immunoprecipitation:** Western blot analyses were performed using specific anti-sera or monoclonal antibodies as described previously (15,17). The expression of β-actin was used as a loading control in immunoblot analyses. 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 (30), and then immunoblotted with anti-HSF1, HDAC6 and p97 antibodies. Immunoprecipitated hsp90 was detected by stripping the blot and immunoblotting with an hsp90 antibody (4,17). Horizontal scanning densitometry was performed on Western blots, following acquisition into Adobe PhotoShop (Adobe Systems Inc., San Jose, CA) and analysis by the NIH Image Program (U.S. National Institutes of Health, Bethesda, MD).

**Confocal microscopy and assessment of autophagy:** Breast cancer cells were grown in poly-lysine coated 4-well chamber slides (BD Biosciences) overnight and then incubated with indicated doses of
drugs. Following this, cells were fixed with 4% paraformaldehyde for 10 minutes, washed, permeabilized with 0.5% Triton-PBS buffer for 5 minutes and stained with anti-p62, LC3B-II or polyubiquitin primary antibodies (14,16). Following three washes with PBS, the slides were incubated with Alexa Fluor 555-conjugated goat-anti-mouse secondary antibodies and Alexa Fluor 488 conjugated goat-anti-rabbit secondary antibodies (Invitrogen). The slides were subsequently washed with PBS and counterstained with DAPI using Vectashield mountant containing DAPI. Imaging was performed using Carl Zeiss LSM-510 meta confocal microscope with a 63X/1.2W objective. Induction of autophagy was assessed by monitoring cells with punctate LC3B-II and p62 staining by confocal immunofluorescent microscopy. Autophagic flux was assessed by the increased accumulation of the autophagic substrates LC3B-II and p62 proteins by immunoblot analyses (as described above), following co-treatment of cultured breast cancer cells with PS and CL, compared to treatment with each agent alone (31).

**Annexin V- TO-PRO-3 staining:** Breast cancer cell lines were exposed to the indicated concentrations of the drugs for 48 hours and harvested. Following washes with PBS, cells were stained with annexin-V FITC (BD Biosciences) and TO-PRO-3 iodide. Apoptotic cells were assessed using an Accuri C6 flow cytometer. Synergistic interactions were assessed using the median dose effect analysis of Chou-Talalay (32). Combination index (CI) for each drug was obtained using the commercially available software CalcuSyn (Biosoft, Ferguson, MO) (17,32).

**Mammosphere formation:** MDA-MB-231 cells were exposed to the indicated concentration of drugs for 16 hours. Following this, cells were washed free of drugs, scraped and counted. Equal number of viable cells from each treatment group (20,000 per condition) were re-suspended in 2 ml of complete mammoCult medium (Stem Cell Technologies) and plated in a 6-well ultra-low adherent dish, as described previously (33,34). The cells were cultured for 7 days and the numbers of mammospheres formed were counted. The experiment was performed in duplicate and repeated three times.

**In vivo MB-231-luciferase cell xenografts and in vivo bioluminescent imaging of the xenografts:** Five million MB-231-luciferase cells were implanted into the mammary fat pads of Non-Obese Diabetic/Severe Combined Immunodeficient (NOD/SCID) mice. Tumors were allowed to grow and were harvested when the volumes reached 100, 200, 1000 and 1500 mm³. The tumors were lysed and used for performing immunoprecipitation and immunoblot analyses. Alternatively, cohorts of mice were
divided into four groups (n=5). Tumors were allowed to grow and the treatment was commenced when the average tumor volume reached approximately 100 mm³. Control mice received DMSO (vehicle). PS was administered at a dose of 10 mg/kg three times a week (Day 1, Day 3 and Day 5). CL was administered at a dose of 10 mg/kg once a week (Day 2). No drugs were administered on Day 6 and Day 7 of the week. Mice receiving the combination therapy received both PS and CL as described above. Treatment was continued for three weeks. Tumor volumes were determined by digital calipers and survival of mice was recorded for each group. Mice were humanely sacrificed when the tumor volume reached 1500 mm³ or when the mice lost more than 15% of their body weight and were unable to ambulate. The day of death was noted and plotted on a Kaplan-Meier plot as described previously (17). Tumor growth or regression was also assessed by performing in vivo bioluminescent imaging of the mice using a Xenogen IVIS 2000 in vivo imaging system (Caliper Life Sciences).

**Statistical Analyses:** Data were expressed as mean ± standard deviation. Comparisons used student’s t test or ANOVA, as appropriate. P values of < 0.05 were assigned significance.

**Results:**

**Size-dependent increase in heat shock response, ER stress and autophagy in the orthotopic mouse xenograft model of MB-231-luciferase:** We first determined whether growing tumor size of TNBC is associated with increased evidence for heat shock response ER stress and autophagy. For this, we utilized the orthotopic model of MB-231-luciferase-expressing cells implanted into the mouse mammary fat pads of NOD/SCID mice. From cohorts of mice with increasing tumor sizes (200, 1000 and 1500 cu mm), evaluated by calipers and bioluminescence imaging (Figure 1A), we harvested the tumors and assessed the expression of the markers of heat shock response, ER stress and autophagy. As shown in Figure 1B, there was a notable increase in the protein expression levels of ER stress markers, e.g., GRP78, p-eIF2α, and the pro-autophagy and pro-death transcription factor CHOP (15-17). We also observed a concomitant induction of the levels of the autophagy markers Vps34, ATG7 and LC3B-II (Figure 1B) (11). This was also associated with a size-dependent (200 versus 1500 cu mm) increase in the intracellular levels of polyubiquitylated proteins as well as disruption of the binding of hsp90 to HDAC6-p97 and HSF1 (Figure 1C). Release of HSF1 from this complex is known to induce the nuclear localization, trimerization, phosphorylation and transcriptional activity of HSF1 (35). Consistent with
this, we observed a tumor size-dependent increase in the levels of hsp70 and hsp90 (Figure 1D). We also observed size-dependent increase in the levels of HSF1 and HDAC6, suggesting that increased levels of these proteins support the dependence of the larger TNBC tumors on the heat shock response and HDAC6-promoted aggresome formation necessary to handle the increased intracellular load of misfolded polyubiquitylated proteins.

**Panobinostat induces apoptosis as well as heat shock response and autophagy in cultured breast cancer cells:** Similar to the heat shock and ER stress response and autophagy observed in vivo in the TNBC tumors, previous reports have demonstrated that, in different cancer cell-types, treatment with pan-HDAC inhibitors can also induce apoptosis, heat shock response, ER stress and autophagy in cancer cells (13,15,17). Therefore, next, we determined whether treatment with PS would exert a similar range of effects in the TNBC MB-231 and SUM159T cells. Figure 2A demonstrates that exposure to PS dose-dependently induced apoptosis in cultured breast cancer cells as evidenced by annexin V and TO-PRO-3 positive cells. Figure 2B demonstrates that PS treatment simultaneously induces autophagy in the TNBC cells, which is characterized by increased intracellular levels of LC3B-II and decline in the p62 levels, consistent with the induction of autophagic flux (31,36). Concomitantly, increase in the levels of Vps34 and Beclin 1 was also observed in both cells lines, with greater effects observed at lower concentrations of PS in SUM159PT cells. PS treatment dose-dependently increased cytosolic autophagosome formation in MB-231 cells, as demonstrated by increase in the punctate distribution of LC3B-II and conversion of a diffuse cytosolic to punctate distribution of p62 (31,36). This resulted in the co-localization of LC3B-II and p62, visualized by confocal immunofluorescence microscopy (Figure 2C). Consistent with this, a decline in the p62 levels was observed not only in the detergent-soluble but also detergent-insoluble fraction of the cytosol (Figure 2D). These findings indicate that PS treatment actively recruits p62 to autophagosomes and through autophagic flux induces the turnover of p62 (21,22,31,36). PS was also observed to dose-dependently disrupt the binding of HSF1 and HDAC6/p97 from hsp90 in SUM159T (not shown) and MB-231 cells (Figure 3A). This was associated with the induction of heat shock response, resulting in increased levels of hsp40 and hsp70, while hsp90 levels increased in SUM159T but not in MB-231 cells (Figure 3B).

**Co-treatment with CL impairs PS-induced autophagic flux.** Since treatment with PS induced autophagy in TNBC cells, we determined whether this would increase their dependence on autophagy
for survival. Therefore, we evaluated the effects of co-treatment with CL on PS-induced autophagy. Figure 4A demonstrates that treatment with PS or CL alone increased the cytosolic levels of polyubiquitylated proteins as well as the punctate accumulation of p62. Consistent with its inhibitory effect on the autophagic flux and lysosome function (12,14,37), co-treatment with CL and PS increased the co-accumulation of p62 and polyubiquitylated proteins in the punctate autophagosomes (Figure 4A). These p62 and polyubiquitylated protein-containing foci are reminiscent of aggresome-like structures that are induced by inhibition of autophagy (38). These findings were supported by the results of the immunoblot analyses of the cell lysates from MB-231 cells, following treatment with PS and/or CL. As shown in Figure 4B, treatment with CL alone increased the levels of LC3B-II and p62, by inhibiting lysosomal acidification and inhibition of autophagy. Co-treatment with CL inhibited PS-mediated decline in p62 levels. Importantly, co-treatment with CL did not significantly increase PS-induced hsp70 and GRP78 levels, which are known to be protective against apoptosis (Figure 4C).

**Co-treatment with CL and PS synergistically inhibits survival of TNBC cells.** We next determined whether co-treatment with CL would be lethal toward PS-treated TNBC cells dependent on autophagy for survival. Figure 5A demonstrates that combined treatment with PS and CL induced synergistic loss of survival of MB-231 and SUM159PT cells. The combination indices were less than 1.0 determined by the isobologram analysis of Chou and Talalay (32). Breast cancer stem cells (CSCs) comprise a phenotypically defined population of cells among the bulk primary and metastatic tumor cells, which are resistant to conventional radiation and chemotherapy and considered to be responsible for the relapse of tumors (39-41). Since breast CSCs are characterized by their ability to give rise to mammospheres under anchorage-independent culture conditions in vitro (39), we determined the effect of PS and/or CL on the mammosphere formation by TNBC cells. Figure 5B demonstrates that treatment with PS or CL alone inhibited mammosphere formation by MB-231 cells. Furthermore, compared to each agent alone, co-treatment with PS and CL caused a significantly greater inhibition of mammospheres formed by MB-231 cells (p<0.001).

**Combined treatment with PS and CL inhibits in vivo TNBC growth and improves survival of mice bearing TNBC xenograft.** We next determined the effect of treatment with PS and/or CL on the growth of orthotopically implanted, luciferase-expressing MB-231 cells in NOD/SCID mice. Mice were divided into four groups and treated with vehicle (DMSO), PS (10 mg/kg, three times a week) or CL (10
mg/kg, once a week) alone, or with a combination of PS and CL. As shown in Figure 6A and 6B, treatment with PS or CL alone significantly inhibited tumor growth (p < 0.01). Results presented are representative of two experiments. Additionally, Figure 6B shows that co-treatment with PS and CL caused greater inhibition of tumor growth, noted as lesser bioluminescence intensity, as compared to treatment with each agent alone. Although by caliper measurements of the tumors, the combined treatment with PS and CL produced the smallest mean tumor volumes, this was not significantly different from those observed following treatment with PS or CL alone (p > 0.05) (Figure 6A). Notably, Kaplan-Meir survival analysis demonstrated that, while the control mice (treated with vehicle alone) exhibited a median survival of 37 days post-implantation, treatment with PS or CL alone improved the median survival of the mice to 42 and 41 days, respectively (Figure 6C). Furthermore, importantly, co-treatment with PS and CL significantly improved the median survival of the mice to 60 days (p < 0.003) (Figure 6C).

**Discussion:**

The present studies for the first time show that treatment with the pan-HDAC inhibitor PS disrupts the binding of hsp90 and HSF1, HDAC6 and p97, thereby eliciting an HSF1-mediated heat shock response in TNBC cells. The dissociation of HSF1 from the HDAC6-hsp90-p97-HSF1 complex is known to cause HSF1 phosphorylation, trimerization, and nuclear translocation, resulting in the transcriptional activation of heat shock proteins (6,35). Consistent with this, treatment with PS induced hsp70 and hsp40 levels in TNBC cells. This is known to be an adaptive response both to reduced chaperone function of hsp90 and to increased levels of intracellular misfolded polyubiquitylated proteins (4,6). PS treatment also inhibited HDAC6 activity, as represented by increase in acetylated α-tubulin levels. This was previously demonstrated to inhibit HDAC6-mediated aggresome formation, which is another adaptive response to proteotoxic stress due to misfolded polyubiquitylated proteins (8,9). Consistent with previous reports, treatment with PS also induced ER stress response in TNBC cells, as represented by induction of GRP78 (15-17). Although not studied here, in a previous report we had demonstrated that PS treatment induces hyperacetylation of GRP78, which may affect its function in the ER, accentuating the ER stress response in breast cancer cells (15).

Increased intracellular accumulation of polyubiquitylated unfolded proteins and ER stress response (UPR) leads to increase in the ER capacity to fold its client proteins, e.g., through the induction of
GRP78 (9,15,42). This was observed here in PS-treated TNBC cells. Alternatively, if the ER stress is protracted, lethal ER stress ensues, which induces apoptosis (16). However, UPR also induces autophagy, for clearing polyubiquitylated protein aggregates and protection against lethal ER stress (42,43). During ER stress, binding of GRP78 to unfolded proteins releases and activates the three mediators of UPR, i.e., PERK (PKR-like eIF2α kinase), ATF6 (activated transcription factor 6) and IRE1 (inositol requiring enzyme 1) (16). Of these, the activities of PERK and ATF6 are involved in induction of autophagy (11,43). Indeed, UPR induces autophagy in hypoxic cells partly by increasing the transcription of the essential autophagy genes microtubule-associated protein 1 light chain 3beta (MAP1LC3B) and autophagy-related gene 5 (ATG5) through transcriptional activation by ATF4 and CHOP, respectively, which are regulated by PERK (18). Our findings clearly demonstrate that during PS-induced ER stress and autophagy in TNBC cells, there is not only an increase in the levels of Vps34, Beclin 1 and LC3B-II, but also co-localization of p62 and LC3B-II (Figures 2B and 2C). The selective autophagic degradation of protein aggregates requires ubiquitin binding receptors such as p62 (9,11), which link simultaneously with ubiquitin and autophagy-specific ubiquitin like modifiers such as LC3B (9,11,22). As has been shown by numerous studies, our findings also show that exposure to PS increased LC3B-II but reduced the levels of p62 in both the detergent soluble and insoluble cellular fractions of TNBC cells, consistent with the degradation of p62 during PS-induced autophagic flux (9,21,22). This indicated that PS-treated TNBC cells would be particularly dependent on PS-mediated heat shock response, UPR and autophagy for survival, making them particularly susceptible to inhibition of autophagy by agents such as CL, which inhibits autophagic flux thereby increasing levels of p62 and LC3B-II (11,12,37). Indeed, treatment with CL alone, while markedly increasing LC3B-II levels, also increased the levels of p62 and its localization with polyubiquitylated proteins in TNBC cells. CL treatment reduced GRP78 without increasing hsp70 levels. Several studies have shown that targeting autophagy where it is promoting survival of cancer cells under stress would achieve selective antitumor efficacy (11,12,14,37). Accordingly, co-treatment with CL significantly inhibited PS-induced autophagic flux, thereby maintaining high levels of LC3B-II and p62 in TNBC cells. This was associated with synergistic apoptosis of TNBC cells following co-treatment with CL and PS. It is noteworthy that, while treatment with CL, more so than PS, alone inhibited the in vitro mammosphere formation by TNBC cells, co-treatment with PS and CL had a profound inhibitory effect on the mammosphere growth. These findings suggest that inhibition of autophagic flux with CL markedly increased the inhibitory effects of PS on TNBC cells with a stem cell phenotype.
A recent proteomic analysis of the autophagy interaction network of basal autophagy has highlighted a network of interacting proteins with extensive connectivity that particularly involved ATG8 interacting with LC3-interacting regions in the ubiquitin binding partner proteins such as p62 (44). Importantly, our findings also demonstrate that within tumors of TNBC cells growing orthotopically in vivo, there is increased accumulation of polyubiquitylated proteins, disruption of hsp90-HSF1-HDAC6-p97 complex, which is associated with heat shock response and increased evidence for ER stress and autophagy (Figure 1). The evidence for the size-dependent increase in the autophagic flux was supported by the attendant decline in p62 levels (21,22). Autophagy in breast cancer cells has also been shown to mitigate metabolic stress and genome damage (45). Additionally, by inducing further stress in orthotopically growing TNBC tumors, treatment with PS would enhance the dependence of the tumors for survival and growth on the adaptive heat shock response, UPR and the ensuing autophagy (9,11,13). This would enable an accentuated anti-tumor activity of co-treatment with CL and PS against TNBC. Our findings confirm that in vivo co-treatment with CL and PS was more effective in reducing the tumor growth and survival of the mice orthotopically implanted with TNBC cells, as compared to treatment with each agent alone. This is consistent with the recent reports where targeting autophagy in conjunction with treatment with HDI was shown to exert superior anti-tumor activity in peripheral nerve sheath tumors (46). It is also noteworthy that combined in vitro treatment with PS and CL was observed to reduce the levels of GRP78 and hsp70 in TNBC cells, which is particularly pertinent because simultaneous reduction in hsp70 and GRP78 synergistically induces cell death of breast cancer cells (47). Recently, hsp70 was shown to promote autophagy and cell survival (48). It is possible that, in the present studies, by reducing the levels of hsp70 and GRP78, while simultaneously inhibiting the protective autophagy induced by PS, co-treatment with CL markedly augmented the lethal effects of PS against the bulk TNBC as well as TNBC stem cells.

The expression of p62 has also been reported to be high in breast cancer cells compared to normal cells (49). Accumulation of p62 in autophagy-defective cells has been shown to increase oxidative stress and cell death (21,22). Our study supports this by demonstrating that, following treatment with PS and CL, increased accumulation of polyubiquitylated proteins that co-localize with the increased p62 foci in the cytosol is associated with increased loss of survival of TNBC cells. This is possibly the result of protein quality control failure and the ensuing cell death due to proteotoxicity (10,11). This appears to be
especially relevant for the stem cell potential of TNBC cells, as measured by their ability to grow as mammospheres in suspension. Consistent with this, co-treatment with PS and CL significantly reduced mammosphere formation by TNBC cells. This observation has far-reaching implications in the eradication of cancer stem cells which are more resistant to chemotherapy (41,50). Furthermore, co-treatment with PS and CL results in significantly improved survival of mice bearing orthotopic TNBC xenografts (Figure 6C). Although not fully explored in this study, it is likely that inhibition of the stem cell population in tumors treated with the combination of PS and CL could also contribute to the longer disease-free survival of MB-231 xenografts. Taken together, our data clearly support the rationale for testing the combination of pan-HDAC inhibitor and autophagy inhibitor as a therapy against the otherwise therapy-resistant and aggressive TNBCs.
REFERENCES


FIGURE LEGENDS

Figure 1: Induction of autophagy in an orthotopic breast tumor model. A. MB-231 cells expressing luciferase were orthotopically implanted into the mammary fat pads of NOD-SCID mice and the tumors were imaged when they reached 100, 200, 1000 and 1500 mm$^3$, using a Xenogen IVIS 2000 in vivo imaging system. The images displayed in panel A are representative of three independent experiments. The numbers beneath the blots are densitometric readings of the protein quantity. B. Cell lysates from the harvested tumors were immunblotted with specific antibodies raised against GRP78, p-eIF2α, eIF2α, CHOP, Vps34, ATG7, LC3B-II and β-actin. C. Hsp90 was immunoprecipitated from tumor lysates at indicated tumor sizes or from cultured MB-231 cells (CC) and immunoblot analyses were performed for HSF1, p97, HDAC6 and hsp90. Levels of polyubiquitylated proteins in the corresponding tumor lysates was assessed by immunoblot analysis (IB) by immunoblotting with antibodies against polyubiquitin (lower panel) D. The expression of HDAC6, heat shock proteins, HSF1, hsp70, hsp90 and acetylated hsp90 (Ac K69/hsp90) in tumors of the indicated sizes was assessed using immunoblot analyses.

Figure 2: PS induces both apoptosis and autophagy in cultured breast cancer cells A. MB-231 and SUM159PT cells were treated with the indicated doses of PS for 48 hours and the percentage of apoptotic cells were assessed by flow cytometry. B. MB-231 and SUM159PT cells were exposed to the indicated doses of PS for 16 hours. Immunoblot analyses were performed for Vps34, Beclin 1, LC3B-II, p62 and β-actin. The numbers beneath the blots are densitometric readings of the protein quantity relative to the control cells. C. MB-231 cells were incubated with PS for 16 hours and the slides were washed, fixed and stained with antibodies against LC3B-II (green) and p62 (red). The slides were imaged using an LSM 510-meta confocal microscope using a 63X/1.2W lens. D. MB-231 and SUM159PT cells were exposed to the indicated doses of PS for 16 hours and the levels of p62 were assessed in the detergent-soluble and detergent-insoluble cell fractions. The expression of β-actin was used as the loading control.

Figure 3: PS elicits a heat shock response in cultured breast cancer cells. A. MB-231 cells were incubated with the indicated doses of PS for 6 hours then hsp90 was immunoprecipitated from the cell lysates and immunoblot analyses were performed for HSF1, p97 and HDAC6. B. MB-231 and
SUM159PT cells were treated with the indicated doses of PS for 16 hours. Immunoblot analyses were performed for hsp90, hsp40, hsp70, acetylated α-tubulin, and β-actin.

**Figure 4: Co-treatment with PS and CL impairs autophagy and accentuates PS-induced p62 foci formation in breast cancer cells.** A. MB-231 cells were exposed to PS and/or CL for 16 hours. Then, the cells were cytospun onto glass slides and immunostained with antibodies against polyubiquitin (green) and p62 (red). The slides were imaged using an LSM 510-meta confocal microscope using a 63X/1.2W lens. **B & C.** MB-231 and SUM159PT cells were exposed to the indicated doses of PS and CL for 16 hours. Immunoblot analyses were performed for p62, LC3B-II, hsp70, GRP78 and β-actin.

**Figure 5: Co-treatment with PS and CL results in synergistic cell death in breast cancer cells and results in a reduced number of mammospheres.** A. MB-231 and SUM159PT cells were exposed to the indicated doses of PS and CL at a constant ratio and the percentage of apoptotic cells were assessed by flow cytometry. Median dose effect and isobologram analyses were performed to obtain combination index (CI) values using the CalcuSyn software. CI values less than 1.0 represent synergistic interaction of the two drugs. **B.** MB231 cells were exposed to the indicated doses of PS and CL for 16 hours. The cells were scraped and washed free of drugs. Mammosphere formation was assessed by plating 20,000 cells per well and followed by incubation in complete mammocult medium in a 37°C incubator. The number of mammospheres formed in each treatment group was counted at the end of 7 days and the plotted as a percent of the untreated control.

**Figure 6: Treatment with PS and CL results in tumor regression and co-treatment with PS and CL improves the survival MB-231 cell xenografts.** A. MB-231-luciferase cells were orthotopically implanted into the mammary fat pads of NOD/SCID mice and the tumors were allowed to develop. Mice were randomized into four groups (n=5 mice per group). Treatment with PS, CL or the combination was commenced three weeks after implantation of cells (average tumor volume of 100 mm³) and continued for three weeks. Tumor volumes were recorded and the average tumor size was plotted for each treatment group. **B.** Tumors were imaged at the end of treatment and panel B shows a representative image from each group of mice. **C.** Kaplan-Meier survival plot for MB-231 xenografts following treatment with PS alone, CL alone, or the combination of PS plus CL.
Figure 1

A) MB-231 xenograft

Tumor size, mm³

100  200  1000  1500

B) MB-231 xenograft

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<tbody>
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<td>100  200  1000  1500</td>
</tr>
<tr>
<td>GRP78</td>
</tr>
<tr>
<td>eIF2α</td>
</tr>
<tr>
<td>eIF2α</td>
</tr>
<tr>
<td>CHOP</td>
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<tr>
<td>Vps34</td>
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<tr>
<td>ATG7</td>
</tr>
<tr>
<td>LC3B-II</td>
</tr>
<tr>
<td>β-actin</td>
</tr>
</tbody>
</table>

C) MB-231 xenograft

Tumor size, mm³

IP: hsp90

IB:

<table>
<thead>
<tr>
<th>Tumor size, mm³</th>
<th>IP: hsp90</th>
</tr>
</thead>
<tbody>
<tr>
<td>100  200  1500</td>
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</tr>
<tr>
<td>HDAC6</td>
<td></td>
</tr>
<tr>
<td>HSF1</td>
<td></td>
</tr>
<tr>
<td>p97</td>
<td></td>
</tr>
<tr>
<td>hsp90</td>
<td></td>
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</tbody>
</table>

D) MB-231 xenograft

<table>
<thead>
<tr>
<th>Tumor size, mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>100  200  1000  1500</td>
</tr>
<tr>
<td>HDAC6</td>
</tr>
<tr>
<td>hsp90</td>
</tr>
<tr>
<td>hsp70</td>
</tr>
<tr>
<td>HSF1</td>
</tr>
<tr>
<td>Ac K69/hsp90</td>
</tr>
<tr>
<td>β-actin</td>
</tr>
</tbody>
</table>

1.0 1.05 1.14 1.28
Figure 3

A

MB-231

IgG 0 10 20 50 nM, PS, 6 hours

IP: hsp90

IB:

HSF1

HDAC6

p97

hsp90

B

MB-231

SUM159PT

0 10 20 50 nM, PS, 16 hours

hsp90

hsp40

hsp70

acetyl- α-tubulin

β-actin
Figure 4

A. MB-231, 16 hours

<table>
<thead>
<tr>
<th>Condition</th>
<th>Polyubiquitin</th>
<th>p62</th>
<th>MERGE</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td><img src="control_polyubiquitin" alt="" /></td>
<td><img src="control_p62" alt="" /></td>
<td><img src="control_MERGE" alt="" /></td>
</tr>
<tr>
<td>20 nM, PS</td>
<td><img src="20_nM_PS_polyubiquitin" alt="" /></td>
<td><img src="20_nM_PS_p62" alt="" /></td>
<td><img src="20_nM_PS_MERGE" alt="" /></td>
</tr>
<tr>
<td>PS + CL</td>
<td><img src="PS_CL_polyubiquitin" alt="" /></td>
<td><img src="PS_CL_p62" alt="" /></td>
<td><img src="PS_CL_MERGE" alt="" /></td>
</tr>
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</table>

B. Western Blot

<table>
<thead>
<tr>
<th>Condition</th>
<th>MB-231</th>
<th>SUM159PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM, PS</td>
<td><img src="0_20_50_0_20_ps_p62" alt="" /></td>
<td><img src="0_20_50_0_20_ps_p62" alt="" /></td>
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<tr>
<td>20 nM, PS</td>
<td><img src="20_20_50_20_ps_p62" alt="" /></td>
<td><img src="20_20_50_20_ps_p62" alt="" /></td>
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<tr>
<td>25 µg/ml CL</td>
<td><img src="25_cl_p62" alt="" /></td>
<td><img src="25_cl_p62" alt="" /></td>
</tr>
</tbody>
</table>

C. Western Blot

<table>
<thead>
<tr>
<th>Condition</th>
<th>MB-231</th>
<th>SUM159PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM, PS</td>
<td><img src="0_20_50_0_20_ps_hsp70" alt="" /></td>
<td><img src="0_20_50_0_20_ps_hsp70" alt="" /></td>
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<tr>
<td>20 nM, PS</td>
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<td><img src="20_20_50_20_ps_hsp70" alt="" /></td>
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<tr>
<td>25 µg/ml CL</td>
<td><img src="25_cl_hsp70" alt="" /></td>
<td><img src="25_cl_hsp70" alt="" /></td>
</tr>
</tbody>
</table>

The images show the effects of various treatments on polyubiquitin and p62 levels in MB-231 cells. The western blot images illustrate the protein levels of LC3B-II and β-actin under different conditions.
Figure 5

A

MB-231

SUM159PT cells

<table>
<thead>
<tr>
<th>PS (nM)</th>
<th>CL (µg/mL)</th>
<th>Fa</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>7.5</td>
<td>0.657</td>
<td>0.498</td>
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<tr>
<td>20</td>
<td>10</td>
<td>0.793</td>
<td>0.450</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>0.83</td>
<td>0.589</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>0.837</td>
<td>0.763</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PS (nM)</th>
<th>CL (µg/mL)</th>
<th>Fa</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>0.453</td>
<td>0.298</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>0.709</td>
<td>0.294</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>0.791</td>
<td>0.335</td>
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<tr>
<td>50</td>
<td>50</td>
<td>0.940</td>
<td>0.237</td>
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</table>

B

% mammospheres (Day 7)

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>50 nM, PS</th>
<th>25 µg/mL, CL</th>
<th>PS + CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-231</td>
<td>100</td>
<td>80</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>
**Figure 6**

**A**

Average Tumor Volume (mm$^3$), week 5 post-implantation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PS</th>
<th>CL</th>
<th>PS + CL</th>
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<tbody>
<tr>
<td>Volume</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**B**

MB-231-luciferase xenograft

**C**

MB231-luciferase xenograft

- Control
- PS
- Chloroquine
- PS + CL

Percent survival vs. Days of Implantation
Molecular Cancer Therapeutics

Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells


Mol Cancer Ther Published OnlineFirst February 24, 2012.

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Access the most recent supplemental material at:
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