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 (HDI) 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 and the autophagy inhibitor chloroquine against human estrogen/progesterone receptor and HER2 (triple)-negative breast cancer (TNBC) cells. Treatment of MB-231 and SUM159PT cells with panobinostat disrupted the hsp90/histone deacetylase 6/HSF1/p97 complex, resulting in the upregulation of hsp. This was accompanied by the induction of enhanced autophagic flux as evidenced by increased expression of LC3B-II and the degradation of the autophagic substrate p62. Treatment with panobinostat also induced the accumulation and colocalization of p62 with LC3B-II in cytosolic foci as evidenced by immunofluorescent confocal microscopy. Inhibition of panobinostat-induced autophagic flux by chloroquine 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 with 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 were observed. Cotreatment with panobinostat and chloroquine resulted in reduced tumor burden and the survival of MB-231 breast cancer xenografts. Collectively, our findings show that cotreatment with an autophagy inhibitor and pan-HDI, for example, chloroquine and panobinostat results in accumulation of toxic polyubiquitylated proteins, exerts superior inhibitory effects on TNBC cell growth, and increases the survival of TNBC xenografts.

Mol Cancer Ther; 11(4); 973–83.

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 multiprotein complex consisting of hsp90, HSF1, histone deacetylase 6 (HDAC6), and p97 (3, 4). This releases and activates HSF1, which induces the hsp, including hsp40, hsp70, and hsp90 (5, 6). The hsp 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 upregulated under conditions in which 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 (HDAC inhibitors), such as vorinostat and panobinostat, 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 panobinostat inhibits aggresome formation and induces endoplasmic reticulum (ER) stress response represented by upregulation of the chaperoneGRP78 and activation of the transcription factors ATF4 and CHOP (14–16). ER stress response and activation of ATF4 and CHOP has also been shown to induce autophagy genes, thereby linking ER stress to autophagy (17).

There are more than 30 autophagy-related (Atg) genes in yeast, which encode proteins that are essential for the execution of autophagy (18). 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, 19). The phagophore expansion and completion of autophagosome formation is mediated by the 2 ubiquitin-like Atg12–Atg5 and LC3-phosphatidylethanolamine (PE) conjugation pathways (11, 19). 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, 19). LC3B-II is degraded by lysosomal hydrolases after the fusion of autophagosomes with lysosomes (11, 19). 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 (20). Following fusion of the autophagosome to lysosome, p62 and polyubiquitylated proteins are degraded (21). When autophagy is inhibited, there is accumulation of p62 and polyubiquitylated proteins (21).

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 (21, 22). Autophagy-deficient mice due to Atg7 loss develop liver tumors and accumulate p62 (23, 24). However, autophagy can also allow tumor cells to survive stress due to hypoxia, nutrient withdrawal, and cancer therapeutic agents (11, 12). On the basis of accumulating evidence for the role of autophagy in promoting tumor survival under stressful conditions, the strategy of abrogating autophagy with chloroquine (and hydroxychloroquine) for promoting tumor regression has been recently tested in preclinical and clinical settings (12, 25). Chloroquine is a lysomotropic agent that inhibits autophagic flux by disturbing lysosome pH and function (12). Because treatment with pan-HDI is known to induce autophagy, in this study, we investigated the molecular mechanism(s) of cell death following cotreatment with panobinostat and chloroquine 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 show that, in vivo, advanced TNBC displays evidence for ER stress and autophagy. We also show that treatment of TNBC cells with panobinostat accentuates ER stress and autophagy, and cotreatment with chloroquine significantly enhances the in vitro and in vivo efficacy of panobinostatin TNBC cells.

Materials and Methods

Reagents and antibodies

Panobinostat was provided by Novartis Pharmaceuticals Inc. Chloroquine was obtained from Sigma-Aldrich. The chemical structures for panobinostat and chloroquine are provided in Supplementary Fig. S1. Anti-phospho-eIF2a, eIF2a, 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 (ATCC; HTB-26). SUM159PT cells were obtained from University of Michigan Human Breast Cancer Cell/Tissue Bank/Asterand. Both cell lines were banked after receipt and passaged for less than 6 months before use in this study. The ATCC and Asterand characterize cell lines using short-tandem repeat polymorphism analysis. MDA-MB-231 (referred to as MB-231 in this article) cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 10% FBS 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 before the performance of the studies described below.

Western blot analyses and immunoprecipitation

Western blot analyses were carried out using specific antisera or monoclonal antibodies as described previously (14, 16). The expression of β-actin was used as a loading control in immunoblot analyses. Data presented are representative of at least 3 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, 16). Horizontal scanning densitometry was done on Western blots, following acquisition into Adobe PhotoShop (Adobe Systems Inc.) and analysis by the NIH Image Program (NIH, Bethesda, MD).

Confocal microscopy and assessment of autophagy
Breast cancer cells were grown in polylysine-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 (15, 26). Following 3 washes with PBS, the slides were incubated with Alexa Fluor 555-conjugated goat-antimouse secondary antibodies and Alexa Fluor 488 conjugated goat-antirabbit secondary antibodies (Invitrogen). The slides were subsequently washed with PBS and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) using Vectashield mountant containing DAPI. Imaging was done 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 immune fluorescent 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 cotreatment of cultured breast cancer cells with panobinostat and chloroquine, compared with 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 fluorescein isothiocyanate (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 and Talalay (32). Combination index (CI) for each pair of drugs was obtained using the commercially available software Calcusyn (Biosoft; refs. 16, 32).

Mammosphere formation
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 resuspended in 2 mL of complete mammmocult medium (STEM Cell Technologies) and plated in a 6-well ultralow 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 done in duplicate and repeated 3 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, 1,000, and 1,500 mm³. The tumors were lysed and used for carrying out immunoprecipitation and immunoblot analyses. Alternatively, cohorts of mice were divided into 4 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 (dimethyl sulfoxide; vehicle). Panobinostat was administered at a dose of 10 mg/kg 3 times a week (days 1, 3, and 5). Chloroquine was administered at a dose of 10 mg/kg once a week (day 2). No drugs were administered on days 6 and 7 of the week. Mice receiving the combination therapy received both panobinostat and chloroquine as described above. Treatment was continued for 3 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 1,500 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 (16). Tumor growth or regression was also assessed by carrying out 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 ± SD. Comparisons used Student t test or ANOVA, as appropriate. P values of less than 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 used 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, 1,000, and 1,500 cu mm), evaluated by calipers and bioluminescence imaging (Fig. 1A), we harvested the tumors and assessed the expression of the markers of heat shock response, ER stress, and autophagy. As shown in Fig. 1B, there was a notable increase in the protein expression levels of ER stress markers, for example, GRP78, p-eIF2α, and the proautophagy and prodeath transcription factor CHOP (14–16). We also observed a concomitant induction of the levels of the autophagy markers Vps34, ATG7, and LC3B-II (Fig. 1B; ref. 11). This was also associated with a size-dependent (200 vs. 1,500 cu mm) increase in the intracellular levels of...
polyubiquitylated proteins as well as disruption of the binding of hsp90 to HDAC6-p97 and HSF1 (Fig. 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 (Fig. 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 shown that, in different cancer cell types, treatment with pan-HDAC inhibitors can also induce apoptosis, heat shock response, ER stress, and autophagy (13, 14, 16). Therefore, next, we determined whether treatment with panobinostat would exert a similar range of effects in the TNBC MB-231 and SUM159PT cells. Figure 2A shows that exposure to panobinostat dose dependently induced apoptosis in cultured breast cancer cells, as evidenced by Annexin V and TO-PRO-3–positive cells. Figure 2B shows that panobinostat 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 panobinostat in SUM159PT cells. Panobinostat treatment dose dependently increased cytosolic...
autophagosome formation in MB-231 cells, as shown 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 colocalization of LC3B-II and p62, visualized by confocal immunofluorescence microscopy (Fig. 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 (Fig. 2D). These findings indicated that panobinostat treatment actively recruits p62 to autophagosomes and through autophagic flux induces the turnover of p62 (20, 21, 31, 36). Panobinostat was also observed to dose dependently disrupt the binding of HSF1 and HDAC6/p97 from hsp90 in SUM159T (not shown) and MB-231 cells (Fig. 3A). This was associated with the induction of heat shock response, resulting in increased levels of hsp40 and hsp70, whereas hsp90 levels increased in SUM159T but not in MB-231 cells (Fig. 3B).

Cotreatment with chloroquine impairs panobinostat-induced autophagic flux

Because treatment with panobinostat induced autophagy in TNBC cells, we determined whether this would increase their dependence on autophagy for survival. Therefore, we evaluated the effects of cotreatment with chloroquine on panobinostat-induced autophagy. Figure 4A shows that treatment with panobinostat or chloroquine 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, 26, 37), cotreatment with chloroquine and panobinostat increased the coaccumulation of p62 and polyubiquitylated proteins in the punctate autophagosomes (Fig. 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 panobinostat and/or chloroquine. As shown in Fig. 4B, treatment with chloroquine alone increased the levels of LC3B-II and p62 by inhibiting lysosomal acidification and inhibition of autophagy. Cotreatment with chloroquine inhibited panobinostat-mediated decline in p62 levels. Importantly, cotreatment with chloroquine did not significantly increase panobinostat-induced hsp70 and GRP78 levels, which are known to be protective against apoptosis (Fig. 4C).

**Cotreatment with chloroquine and panobinostat synergistically inhibits survival of TNBC cells**

We next determined whether cotreatment with chloroquine would be lethal toward panobinostat-treated TNBC cells dependent on autophagy for survival. Figure 5A shows that combined treatment with panobinostat and chloroquine 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 (CSC) 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). Because 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 panobinostat and/or chloroquine on the mammosphere formation by TNBC cells. Figure 5B shows that treatment with panobinostat or chloroquine alone inhibited mammosphere formation by MB-231 cells. Furthermore, compared with each agent alone, cotreatment with panobinostat and chloroquine caused a significantly greater inhibition of mammospheres formed by MB-231 cells (*P* < 0.001).

**Combined treatment with panobinostat and chloroquine inhibits *in vitro* TNBC growth and improves survival of mice bearing TNBC xenograft**

We next determined the effect of treatment with panobinostat and/or chloroquine on the growth of orthotopically implanted, luciferase-expressing MB-231 cells in NOD/SCID mice. Mice were divided into 4 groups and treated with vehicle (DMSO), panobinostat (10 mg/kg, 3 times a week) or chloroquine (10 mg/kg, once a week) alone, or with a combination of panobinostat and chloroquine. As shown in Fig. 6A and B, treatment with panobinostat or chloroquine alone significantly inhibited tumor growth (*P* < 0.01). Results presented are representative of 2 experiments. In addition, Fig. 6B shows that cotreatment with panobinostat and chloroquine caused greater inhibition of tumor growth, noted as lesser bioluminescence intensity, as compared with treatment with each agent alone. Although by caliper measurements of the tumors, the combined treatment with panobinostat and chloroquine produced the smallest mean tumor volumes, this was not significantly different from those observed following treatment with panobinostat or chloroquine alone (*P* > 0.05; Fig. 6A). Notably, Kaplan–Meir survival analysis showed that, although the control mice (treated with vehicle alone) exhibited a median survival of 37 days postimplantation, treatment with panobinostat or chloroquine alone improved the median survival of the mice to 42 and 41 days, respectively (Fig. 6C). Furthermore, importantly, cotreatment with panobinostat and chloroquine significantly improved the median survival of the mice to 60 days (*P* < 0.003; ref. Fig. 6C).

**Discussion**

The present studies for the first time show that treatment with the pan-HDAC inhibitor panobinostat 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 hsp (6, 35). Consistent with this, treatment with panobinostat 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). Panobinostat treatment also inhibited HDAC6 activity, as represented by increase in acetylated α-tubulin levels. This was previously shown
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 panobinostat also induced ER stress response in TNBC cells, as represented by induction of GRP78 (14–16). Although not studied here, in a previous report we had shown that panobinostat treatment induces hyperacetylation of GRP78, which may affect its function in the ER, accentuating the ER stress response in breast cancer cells (14).

Increased intracellular accumulation of polyubiquitylated unfolded proteins and ER stress response (UPR) leads to increase in the ER capacity to fold its client proteins, for example, through the induction of GRP78 (9, 14, 42). This was observed here in panobinostat-treated TNBC cells. Alternatively, if the ER stress is protracted, lethal ER stress ensues, which induces apoptosis (15). Our findings clearly show that during panobinostat-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 colocalization of p62 and LC3B-II (Fig. 2B and C). 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 panobinostat increased LC3B-II but reduced the levels of p62 in both the detergent-soluble and detergent-insoluble cellular fractions of TNBC cells, consistent with the degradation of p62 during panobinostat-induced autophagic flux (9, 20, 21). This indicated that panobinostat-treated TNBC cells would be particularly dependent on panobinostat-mediated heat shock response, UPR and autophagy for survival, making them particularly susceptible to inhibition of autophagy by agents such as chloroquine, which

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\text{PERK (PKR-like eIF2\alpha kinase), ATF6 (activated transcription factor 6), and IRE1 (inositol requiring enzyme 1; ref. 15).}
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\text{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 (17). Our findings clearly show that during panobinostat-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 colocalization of p62 and LC3B-II (Fig. 2B and C). The selective autophagic degradation of protein aggregates requires ubiquitin-binding receptors such as p62 (9, 11), which link simultaneously with ubiquitin.
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Figure 4. Cotreatment with panobinostat and chloroquine impairs autophagy and accentuates panobinostat-induced p62 foci formation in breast cancer cells. A, MB-231 cells were exposed to panobinostat and/or chloroquine 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 60X/1.2W lens. B and C, MB-231 and SUM159PT cells were exposed to the indicated doses of panobinostat and chloroquine for 16 hours. Immunoblot analyses were done for p62, LC3B-II, hsp70, GRP78, and \( \beta \)-actin. CL, chloroquine; PS, panobinostat.
inhibits autophagic flux, thereby increasing levels of p62 and LC3B-II (11, 12, 37). Indeed, treatment with chloroquine alone, while markedly increasing LC3B-II levels, also increased the levels of p62 and its localization with polyubiquitylated proteins in TNBC cells. Chloroquine 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, 26, 37). Accordingly, cotreatment with chloroquine significantly inhibited panobinostat-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 cotreatment with chloroquine and panobinostat. It is noteworthy that, while treatment with chloroquine, more so than panobinostat, alone inhibited the in vitro mammosphere formation by TNBC cells, cotreatment with panobinostat and chloroquine had a profound inhibitory effect on the mammosphere growth. These findings suggest that inhibition of autophagic flux with chloroquine markedly increased the inhibitory effects of panobinostat 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 show that within tumors of TNBC cells growing orthotypically 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 (Fig. 1). The evidence for the size-dependent increase in the autophagic flux was supported by the attendant decline in p62 levels (20, 21). Autophagy in breast cancer cells has also been shown to mitigate metabolic stress and genome damage (45). In addition, by inducing further stress in orthotypically growing TNBC tumors, treatment with panobinostat 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 antitumor activity of cotreatment with chloroquine and panobinostat against TNBC. Our findings confirm that in vivo cotreatment with chloroquine and panobinostat was more effective in reducing the tumor growth and survival of the mice orthotypically implanted with TNBC cells, as compared with treatment with each agent alone. This is consistent with the recent reports in which targeting autophagy in conjunction with treatment with HDI was shown to exert superior antitumor activity in peripheral nerve sheath tumors (46). It is also noteworthy that combined in vitro treatment with panobinostat and chloroquine 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 panobinostat, cotreatment with chloroquine markedly augmented the lethal effects of panobinostat 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 with normal cells (49). Accumulation of p62 in autophagy-defective cells has been shown to increase oxidative stress and cell death (20, 21). Our study supports this by showing that, following treatment with panobinostat and chloroquine, increased accumulation of polyubiquitylated proteins that colocalize 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 seems 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, cotreatment with panobinostat and chloroquine significantly reduced mammosphere formation by TNBC cells. This observation has far-reaching implications in the eradication of cancer stem cells that are more resistant to chemotherapy (41, 50). Furthermore, cotreatment with panobinostat and chloroquine results in significantly improved survival of mice bearing orthotopic TNBC xenografts (Fig. 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 panobinostat and chloroquine 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Received December 6, 2011; revised January 23, 2012; accepted February 10, 2012; published OnlineFirst February 24, 2012.
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Rao et al.


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Mol Cancer Ther 2012;11:973-983. Published OnlineFirst February 24, 2012.

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