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Addition of a histone deacetylase inhibitor redirects tamoxifen-treated breast cancer cells into apoptosis, which is opposed by the induction of autophagy

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Abstract

Modulation of estrogen signaling is one of the most successful modalities for the treatment of estrogen receptor (ER)-positive breast cancer, yet de novo and acquired resistance are frequent. Recent data suggests that the induction of autophagy may play a considerable role in promoting tumor cell survival and resistance to anti-estrogen therapy. Hence, bypassing autophagy may offer a novel strategy to enhance the anti-tumor efficacy of anti-estrogens. Histone deacetylases (HDAC) are involved in the regulation of steroid hormone receptor mediated cell signaling and their inhibition potentiates the anti-tumor effects of anti-estrogens. However, the mechanism underlying this anti-tumor activity is poorly understood. In this report, we show that the addition of an HDAC inhibitor redirects the response of ER-positive breast cancer cells when treated with tamoxifen from growth arrest to apoptotic cell death. This redirection requires functional ER signaling and is mediated by a depletion of Bcl-2 and an induction of Bax and Bak, manifesting in cytochrome C release and PARP cleavage. With combined treatment, a subpopulation of cells are refractory to apoptosis and exhibit a strong induction of autophagy. Inhibition of autophagy in these cells, using siRNA directed against Beclin-1 or treatment with chloroquine, further promotes the induction of apoptosis. Thus, supporting prior reports that autophagy acts as a survival mechanism, our findings demonstrate that HDAC and autophagy inhibition directs autophagy-protected cells into apoptotic cell death, which may impair development of tamoxifen resistance.

Keywords

breast cancer; histone deacetylase; anti-estrogen therapy; estrogen receptor; autophagy; apoptosis

INTRODUCTION

Estrogen-receptor (ER) signaling is a key regulator of gene transcription and a major driver of tumorigenesis in breast cancer. The modulation of ER signaling is the preferred strategy in the treatment of hormone-sensitive breast cancer. The selective ER modulator tamoxifen is the most commonly administered anti-estrogen for pre-menopausal patients. Tamoxifen competes with estrogen for binding to the ER and alters the function of ER complexes and target gene transcription. Although more than two-thirds of breast cancers express ER, fewer than half respond to anti-estrogen therapy. Furthermore, patients initially responding to anti-estrogen therapy will eventually become refractory and exhibit a more aggressive disease with a poorer prognosis, illustrating the high likelihood of hormone therapy resistance [1].

Acetylation is a key mediator of estrogen signaling, regulating the transcription of ER as well as the stability of ER complexes. Acetylation is controlled by histone acetyltransferases and histone deacetylases (HDACs), which add and remove acetyl groups, respectively, from lysine residues of both histone and non-histone targets [2]. The aberrant expression of HDACs has been associated with many malignancies, including gastric, lung, esophageal, colon, prostate, endometrial, and breast cancer, indicating their potential as therapeutic targets [3–11]. Indeed, HDAC inhibitors have emerged as established anti-cancer agents, with two, vorinostat and romidepsin, approved for the treatment of cutaneous T-cell lymphoma [12–14].

Several reports suggest that HDAC inhibitors may enhance the cytotoxicity of anti-estrogen therapy for the treatment of breast cancer. A number of *in vitro* studies have shown that HDAC inhibitors can re-establish the expression of ER in ER-negative breast cancer cell lines. In these cells, re-expression was accompanied with ER transcriptional activity and sensitivity to tamoxifen treatment [15–17]. We have shown that when breast cancer cells are treated with HDAC inhibitors in combination with tamoxifen, apoptosis is induced and cell death is potentiated [18]. Currently, the effectiveness of this combination is being evaluated in a phase II clinical trial of patients with locally advanced or invasive breast cancer, who have previously been treated with hormonal therapy (clinicaltrials.gov; NCT00365599). Although promising, the mechanism underpinning the effectiveness of this combination therapy is unclear.

Recent reports point to autophagy as a potential mechanism of emerging tamoxifen resistance [19, 20]. Autophagy is a cellular process of self-consumption that is activated in response to stress. Within the cytoplasm, autophagic vesicles are formed, which engulf cellular macromolecules and organelles. These vesicles fuse with hydrolase containing lysosomes and the contents are digested. This mechanism allows the cell to reclaim molecular building blocks by digesting cellular components, including damaged organelles, to survive during periods of starvation or toxic insult. Hence, cancer cells may utilize autophagy during times of metabolic stress to provide the tumor cell with essential nutrients and to prevent the induction of apoptosis by digesting mitochondria that could release pro-apoptotic inducers. However, prolonged induction of autophagy can lead to cell death by activating apoptosis or by massive autophagic vacuolarization [21].

Several reports suggest that induction of autophagy in response to anti-estrogen treatment may contribute to the emergence of tamoxifen resistant breast cancer [22, 23]. In this study, we sought to determine the role of tamoxifen-induced autophagy and the induction of apoptosis achieved with the co-administration of an HDAC inhibitor in ER-positive breast cancer cells. We demonstrate that autophagy is induced in response to combined treatment with an HDAC inhibitor and tamoxifen, as a result of Bcl-2 down-regulation and Beclin-1 up regulation. However, a concomitant induction of the pro-apoptotic drivers Bax and Bak switches the primary response of cells treated with tamoxifen from anti-proliferative to apoptotic. However, a small population of cells remains refractory to apoptotic induction, due to autophagy-associated survival. Thus, to avoid the emergence of resistant clones, our work suggests inhibition of autophagy should be considered when adding an HDAC inhibitor to tamoxifen treatment.

MATERIAL AND METHODS

Chemicals and antibodies

Valproic acid, chloroquine, fulvestrant and 17 β -estradiol (E2) were purchased from Sigma-Aldrich (St. Louis, MO). Vorinostat (suberoylanilide hydroxamic acid) was provided by Aton Pharma (Whitehouse Station, NJ). 4-OH-tamoxifen was purchased from Calbiochem

(San Diego, CA). ER α and PR antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Bcl-2, Bax, Bak and PARP antibodies were purchased from Cell Signaling technology (Danvers, MA). Beclin-1 antibody was purchased from ProSci Inc. (Poway, CA). HDAC2 antibody was purchased from Upstate Biotechnology (Chicago, IL). LC3 antibody was purchased from Novus Biologicals (Littleton, CO). Cytochrome C antibody was provided with the ApoAlert Cell Fractionation kit (Clontech, Mountain View, CA). GAPDH antibody was purchased from Chemicon (Temecula, CA).

Cell Culture

All cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Fisher Scientific, Atlanta, GA) with 10% heat inactivated fetal bovine serum (FBS, Sigma-Aldrich), 2 mM glutamine, and 50 unit/mL penicillin and 50 μ g/mL streptomycin (Fisher Scientific). Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C. For experiments evaluating the effect of estrogen concentration, cells were maintained in phenol red free DMEM supplemented with 10% dextran-charcoal stripped FBS (Invitrogen, Carlsbad, CA). Prior to the initiation of these experiments, cells were conditioned by growing them in the absence of estrogen 48 hours.

siRNA Depletion

siRNA duplexes for ER α (ID#42835) and Beclin-1 (sc-29797) were purchased from Ambion (Austin, TX) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Cells were transfected by nucleofection using the Nucleofector transfection kit according to the manufacturer's recommendations (Amaxa, Gaithersburg, MD). Briefly, cells (4×10^6) were suspended in 0.1 mL electroporation buffer containing 1 μ M siRNA and pulsed with a cell line specific protocol as described by the manufacturer. Pulsed cells were suspended in 0.5 mL complete media without antibiotics and incubated at 37°C prior to seeding. Experiments were conducted as indicated the following day. The *Silencer* negative control #2 siRNA (Ambion, ID#4613), a nonsense siRNA duplex, was used as a control.

Cell Proliferation, Viability, and Apoptosis Assays

5×10^3 cells/well were seeded in 96-well plates and drugged the following day as indicated. Each treatment was conducted at least in triplicate. At the indicated time, cell proliferation was assayed using Celltiter 96 AQueous one solution as per manufacturer's instructions (Promega, Madison, WI). Absorbance was measured at 492 nm. Cell viability was determined by dye exclusion assay. Briefly, adherent and floating cells were combined and trypan blue solution (Hyclone, Logan, UT) was added 1:1 (v/v) and incubated for 1 minute at RT. Cells excluding dye were scored as viable. A minimum of 100 cells were scored for each treatment, which was conducted at least in triplicate. To assay for apoptosis, cells were harvested and washed in PBS. Cell nuclei were stained with 0.5 μ g/mL bis-benzimide. Using fluorescent microscopy, cells were scored as apoptotic when nuclear chromatin appeared condensed or fragmented. Two hundred cells were counted per experiment in at least six different fields.

Western Blot Analysis

Cells were harvested, washed with ice-cold PBS, and solubilized using SDS lysis buffer (0.1% SDS, 1% triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, Halt Protease and Phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL), and 1 mM PMSF) by passing the cell suspension through a 20-gauge syringe needle. The lysate protein concentration was determined by Bradford assay. Protein was separated by SDS-PAGE using 4–12% Bis-tris NuPage gels (Invitrogen) and transferred to nitrocellulose membranes

for 1 hour at 100 V at 4°C. Membranes were blocked in tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk (w/v) for 1 hour at room temperature (RT), followed by incubation with the indicated primary antibody (1:1000 v/v) in TBST with 5% nonfat milk, overnight at 4°C. Membranes were washed thrice for 10 minutes with TBST, followed by incubation with the appropriate secondary antibody (1:10000) in TBST with 5% nonfat milk for 1 hour at RT. Protein was visualized by chemiluminescent autoradiography using the Amersham ECL plus detection system (GE Healthcare, Buckinghamshire, UK).

Cell fractionation

The cytoplasmic fraction was separated from mitochondria using the ApoAlert Cell Fractionation Kit according to the manufacturer's protocol (Clontech, Mountain View, CA). Briefly, $\sim 3 \times 10^7$ cells were harvested by trypsinization, washed with ice-cold PBS, followed by washing with Cell Wash Buffer. Washed cells were incubated in Cell Fractionation buffer on ice for 10 minutes. Cells were then dounce homogenized, 50 strokes. Cell homogenate was centrifuged at $700 \times g$ for 10 minutes at 4°C to pellet debris. The supernatant was centrifuged at $10000 \times g$ for 25 minutes at 4°C to pellet mitochondria. The supernatant was collected and saved as the cytoplasmic fraction. Cytoplasmic cytochrome C was quantified by western blot.

RESULTS

HDAC inhibitors increase both apoptosis and autophagy in tamoxifen treated breast cancer cells

Our previous work showed that the addition of an HDAC inhibitor potentiated the effects of tamoxifen in ER-positive breast cancer cells [18]. Forty-eight hours following co-administration of an HDAC inhibitor (*e.g.* valproic acid or vorinostat) and tamoxifen (10 μ M), T47D and MCF-7 cells exhibit a substantial increase in apoptotic nuclei and cell death compared to untreated cells and cells treated with either agent alone. (Supplemental Figure 1 and data not shown). Tamoxifen is a known inducer of autophagy in breast cancer cells and recently has been implicated in the development of tamoxifen resistance. This raised the possibility that the synergy observed with the addition of an HDAC inhibitor may in part be due to inhibition of autophagy by HDAC inhibitors. To evaluate this possibility, T47D and MCF-7 breast cancer cells were treated with VPA and tamoxifen and examined for the induction of apoptosis and autophagy over time measuring PARP cleavage and LC3 (specific to autophagosomes) (Figure 1A and data not shown). As seen previously, tamoxifen treatment was associated with an increase of LC3, yet therapeutic doses of VPA or other HDAC inhibitors (vorinostat and panobinostat) did not induce the expression of LC3 by themselves. However, the addition of VPA to tamoxifen was associated with LC3 induction beyond the effects seen with tamoxifen alone. A notable increase in LC3 was observed following 8 hours treatment and further increased over time. The anti-tumor activity of tamoxifen has been shown previously to be primarily anti-proliferative rather than apoptotic. Consistent with these findings, neither tamoxifen nor VPA treatment alone was associated with PARP cleavage (Figure 1) or apoptotic nuclei (supplemental figure). However, when treated with the combination, cells exhibited significant PARP cleavage by 48 hours, which increased further with time.

To determine whether these findings were specific to valproic acid, we tested whether other HDAC inhibitors increase the autophagy observed with tamoxifen treatment. T47D cells were treated with the pan-HDAC inhibitor, vorinostat (1 μ M) or panobinostat (30 nM), tamoxifen (10 μ M), or the combination of each HDAC inhibitor with tamoxifen. Following 48 hours treatment, LC3 levels were evaluated by western blot (Figure 1B). Alone, LC3 levels in cells treated with either HDAC inhibitor were comparable to untreated cells, while

tamoxifen treatment induced a modest increase in LC3. However, when combined with tamoxifen, both vorinostat and panobinostat elicited increased LC3 levels compared to cells treated with tamoxifen alone, with the panobinostat combination yielding a more robust increase.

Together, these results demonstrate that when combined with tamoxifen, HDAC inhibitors potentiate tamoxifen-induced LC3 expression, a component specific to autophagosomes and a hallmark of autophagy. Furthermore, LC3 induction precedes apoptosis induced by the addition of an HDAC inhibitor to tamoxifen.

In the presence of an HDAC inhibitor, cells undergo apoptosis despite tamoxifen-induced autophagy

The evaluation of LC3 induction and PARP cleavage in cells treated with an HDAC inhibitor and tamoxifen suggested a temporal order of induction, with autophagy preceding apoptosis. To determine whether autophagy induction is a ubiquitous event that precedes apoptosis in all cells or whether autophagy and apoptosis are independent processes occurring in different cell populations, we treated T47D cells with VPA (0.5 mM) and tamoxifen (10 μ M) and evaluated LC3 and PARP cleavage over time by flow cytometry (Figure 2). Thus, the induction of both autophagy and apoptosis could be examined in individual cells. Following 24 hours treatment with tamoxifen or tamoxifen + VPA, 4–6% of cells exhibited elevated LC3 relative to untreated cells, while PARP cleavage was negligible. This trend was similar at 48 hours. By 96 hours however, 11.5% and 12.2% of cells expressed increased LC3 in the tamoxifen and tamoxifen + VPA treatment respectively. However, at this time point, apoptosis measured by cleaved PARP was evident in 21% of the cells treated with the combination, while with tamoxifen treatment alone the percentage of cells with increased PARP cleavage was comparable to untreated cells. Consistent with western blot analysis, treatment with VPA alone did not significantly affect the percentage of cells expressing elevated levels of LC3 or cleaved PARP. Surprisingly, only 5–7% of cells co-expressed of LC3 and cleaved PARP when treated with the combination of VPA and tamoxifen. Taken together, these results do not support a temporal progression from autophagy to apoptosis when cells are treated with an HDAC inhibitor and tamoxifen. Rather, the presence of an HDAC inhibitor evokes the induction of apoptosis in cells exposed to tamoxifen despite the induction of autophagy as a survival strategy.

HDAC inhibitors and tamoxifen induce autophagy in part through ER signaling

Tamoxifen is a selective ER modulator that competes with estrogen for ER binding. The therapeutic efficacy of tamoxifen is thought to be the result of altered ER mediated gene transactivation [1, 24]. Thus, we sought to examine the effect of modulating ER signaling on the observed induction of autophagy with combined HDAC inhibitor and tamoxifen treatment. We first evaluated the effect of high and low dose estrogen on the induction of autophagy. For these experiments, T47D cells were maintained in estrogen stripped media, to which no or 1 nM estradiol was added together with VPA (0.5 mM), tamoxifen (10 μ M), or the combination for 48 hours (Figure 3A). In the absence of additional estradiol, both tamoxifen only treatment and combined treatment with tamoxifen and VPA induced autophagy. However, the marked difference in LC3 expression between these two conditions, seen when estrogen is not stripped from the growth medium, does not appear to be as pronounced when estrogen is depleted. When grown in the presence of high estradiol (1 nM), LC3 induction by tamoxifen alone is substantially reduced, comparable to untreated and VPA treated cells. However, the presence of high estrogen does not substantially abrogate LC3 induction when cells are treated with the combination of VPA and tamoxifen. As previously shown [25], treatment with tamoxifen, regardless of estrogen level, stabilizes the ER. These results suggest that tamoxifen stimulated autophagy is mediated through ER

signaling. With the addition of an HDAC inhibitor, the competitive inhibitory effect of estrogen on autophagy induction is overcome.

We next sought to determine whether combining ER depletion with an HDAC inhibitor could mimic the observed induction of autophagy. To this end, we treated T47D cells with VPA (0.5 mM), the selective ER down regulator fulvestrant (0.1 μ M), or the combination for 48 hours in the presence low and high estrogen levels (Figure 3B). As expected, cells treated with fulvestrant or fulvestrant and VPA exhibited a substantial decrease in ER at both low and high estrogen levels compared to VPA alone or untreated cells. However, an increase in LC3 was not observed. To further explore the importance of the ER for the induction of autophagy, we depleted ER by siRNA in T47D cells and treated them with VPA (0.5 mM), tamoxifen (10 μ M), or the combination for 48 hours (Figure 3C). In support of the experiment with fulvestrant, we found that cells treated with tamoxifen or the combination of tamoxifen and VPA had reduced induction of LC3 when ER was depleted compared to cells transfected with a scramble siRNA control. The requirement of ER for tamoxifen and VPA to fully induce autophagy suggests that this response would not be seen in ER-negative breast cancer cells. To test this hypothesis, we treated the ER-negative MDA-MB-231 cell line with VPA (0.5 mM), tamoxifen (10 μ M) or the combination for 48 hours (Figure 3D). Cells treated with tamoxifen or tamoxifen and VPA exhibited a slight increase in LC3 compared to untreated cells, but not comparable to that seen with ER-positive T47D and MCF-7 cell lines (Figure 1 and data not shown). Together, these results argue that the full induction of autophagy seen with tamoxifen and an HDAC inhibitor requires tamoxifen mediated ER signaling, and not simply the inhibition or degradation of the ER.

Treatment with tamoxifen and an HDAC inhibitor alters the balance of autophagy and apoptosis inhibitors and their drivers

Several studies have shown that tamoxifen treatment may induce autophagy in ER-positive breast cancer cells, possibly contributing to the development of anti-estrogen resistance [19, 22, 23]. This is believed to be in part the result of up-regulating Beclin-1 mRNA and its protein product, an essential component of the autophagic program that when over-expressed can drive production of autophagosomes [26]. Furthermore, it has been shown that the induction of autophagy can effectively inhibit apoptosis. Several groups have postulated that this is due to induction of apoptosis inhibitors, such as BCL2, and depression of apoptosis inducers, such as Bax. To define the link between autophagy and apoptosis, we examined the effects of HDAC inhibition on the balance of autophagy and apoptosis inducers and inhibitors.

As HDACs are key regulators of gene expression, we hypothesized that their inhibition may further result in increased Beclin-1 expression and thus contribute to the observed induction of autophagy. To test this hypothesis, we treated T47D cells with VPA (0.5 mM), tamoxifen (10 μ M), or the combination for 48 hours and harvested cells to evaluate Beclin-1 protein and RNA levels (Figure 4A and B). As previously shown, tamoxifen treated cells had both increased Beclin-1 protein and RNA compared to untreated cells. Cells treated with VPA also had increased Beclin-1 protein, less robust than with tamoxifen, however, RNA levels were unchanged compared to untreated cells. In cells treated with the combination of VPA and tamoxifen, the induction of Beclin-1 exceeded that of either treatment alone. Thus, the effect of VPA on Beclin-1 is predominantly post-translational, as treatment with the combination does not significantly effect Beclin-1 mRNA compared to treatment with tamoxifen alone.

When ER was depleted by siRNA, induction of Beclin-1 protein was substantially reduced in cells treated with tamoxifen and the combination of VPA and tamoxifen compared to

siRNA scramble controls (Figure 3C). Together these results suggest that tamoxifen induces Beclin-1 expression, at least in part, through increased transcription mediated by ER signaling, while VPA acts on Beclin-1 protein stability resulting in a more than additive interaction.

Pro-autophagic signaling of Beclin-1 is regulated by Bcl-2, which binds to the BH3 domain of Beclin-1 through its BH3 binding groove, inhibiting autophagy signaling. Perturbing this interaction, through stress response pathways or by other means, results in autophagy induction [27]. Thus, we measured Bcl-2 protein levels following treatment with VPA (0.5 mM), tamoxifen (10 μ M), or the combination for 48 hours (Figure 4 C). Indeed, when treated with the combination of VPA and tamoxifen, a substantial decrease in Bcl-2 protein was observed. Treatment with either VPA or tamoxifen alone resulted in a negligible change in Bcl-2 protein level.

In addition to regulating autophagy through the inhibition of Beclin-1, Bcl-2 also inhibits the pro-apoptotic function of Bax and Bak. When inhibition is relieved, Bax and Bak act to compromise mitochondrial membrane potential, leading to cytochrome C release into the cytosol where it subsequently promotes activation of execution caspases and cellular digestion [28, 29]. Elevated levels of Bax and Bak, relative to Bcl-2 and Bcl-2 like apoptotic inhibitors, can also lead to cytochrome C release and apoptosis. To determine whether combining an HDAC inhibitor and tamoxifen effect levels of these downstream pro-apoptotic proteins, we evaluated their expression in T47D cells by western blot following 48 hours treatment with VPA (0.5 mM), tamoxifen (10 μ M), or the combination (Figure 4D and E). The combination resulted in both increased Bax and Bak compared to untreated cells. Elevated levels of Bax induced by the combination of VPA and tamoxifen, while either drug alone had negligible effects on Bak levels compared to untreated cells. To determine whether the drug combination further results in compromised mitochondria and cytochrome C release, we fractionated the cytosol from the mitochondria in these cells and measured cytochrome C in the cytosol by western blot (Figure 4F). Compared to untreated cells, only cells treated with the combination of VPA and tamoxifen exhibited increased cytochrome C levels, consistent with our observation that only the combination results in PARP cleavage (Figure 1A).

Together, these results suggest that with the addition of an HDAC inhibitor, tamoxifen treated cells exhibit enhanced autophagic and apoptotic response. In part, this is due to perturbing the balance between the master inhibitor, Bcl-2, and downstream drivers of autophagy, Beclin-1, and apoptosis, Bax and Bak.

Autophagy inhibits the cytotoxicity of VPA and tamoxifen in T47D cells

It has recently been demonstrated that when ER-positive breast cancer cells are treated with tamoxifen, autophagy is induced. In these cells, inhibition of the autophagic program increases cytotoxicity and thus suggests autophagy is promoting survival in response to the insult. However, cells experiencing prolonged elevated autophagy may undergo autophagic cell death. Therefore, we sought to determine whether VPA and tamoxifen induced autophagy was contributing to or inhibiting the cytotoxicity of this combination. First, we evaluated the effect of combining chloroquine with VPA and tamoxifen. Chloroquine preferentially accumulates in the lysosome, raising the pH, and inhibiting subsequent fusion with autophagosomes and degradation of their contents [30]. T47D cells were treated with VPA (0.5 mM), tamoxifen (10 μ M), or the combination and either 0 or 10 μ M chloroquine. In cells treated with chloroquine, autophagosome turnover was inhibited, exhibited by substantially elevated LC3 levels for all treatments compared to cells not treated with chloroquine (Figure 5A). An assay of cell viability revealed that treatment with chloroquine increased cytotoxicity of tamoxifen when administered alone (12% vs. 43% cell death) or

the combination of VPA and tamoxifen (39% vs. 78% cell death) (figure 5B). To more directly assess the role of autophagy, Beclin-1 was depleted by siRNA in T47D cells prior to treatment with VPA and tamoxifen (Figure 5C). Consistent with chloroquine inhibition, when Beclin-1 was depleted, cell death increased nearly 20% with VPA and tamoxifen treatment compared to cells transfected with scramble siRNA and treated with VPA and tamoxifen (Figure 5D).

The findings from these experiments suggest that cells in response to tamoxifen exhibit growth arrest, while a subset of cells induce autophagy in an attempt to escape the effects of tamoxifen. Unlike tamoxifen alone, the addition of an HDAC inhibitor can effectively induce apoptotic cell death by synergistic depletion of Bcl-2 and up-regulation and activation of pro-apoptotic genes such Bax and Bax. However, our data further suggests that a subpopulation of cells may protect themselves from apoptosis by autophagic induction. To avoid the emergence of a resistant cell subpopulation, autophagy must be inhibited. Thus, adding an autophagy inhibitor to an HDAC inhibitor and tamoxifen may represent a more effective treatment regimen for ER-positive tumors.

DISCUSSION

The function of autophagy in cancer is complex, able to both promote and inhibit tumorigenesis depending on the cellular context. Evidence suggests that autophagy functions as a tumor-suppressor in normal tissue and is commonly found defective in several cancers, including breast [31]. However, autophagy is also often found up regulated, thought to allow cancer cells to survive the metabolic stress of the tumor hypoxic microenvironment and demands of sustained cell division [32]. Autophagy has further been shown to promote survival in various cancer cells following treatment with a myriad of chemotherapeutics [33, 34], including breast cancer cells treated with tamoxifen [35]. We have previously shown that the addition of an HDAC inhibitor potentiates the cytotoxicity of tamoxifen in ER-positive breast cancer cells [18]. We hypothesized that HDAC inhibition may potentiate tamoxifen by counteracting tamoxifen-induced autophagy in these cells. Surprisingly, rather than inhibiting autophagy, in this study we found the addition of an HDAC inhibitor to tamoxifen resulted in an initial increase in autophagosomes as measured by LC3, followed by considerable apoptosis.

Evaluation of cells by flow cytometry revealed that autophagic induction is limited to a minor population of cells, while a more significant population of cells initiate apoptosis when treated with the combination. The percentage of cells exhibiting induced autophagy increased only modestly when treated with tamoxifen in combination with an HDAC inhibitor compared to tamoxifen alone, suggesting addition of an HDAC inhibitor increases autophagy within this minor population and not across all cells. When autophagy was inhibited in cells treated with an HDAC inhibitor and tamoxifen, cell death was enhanced. Thus, autophagy in these cells is promoting survival. We believe the promotion of survival within this minor population may be an important factor in enabling the development of tamoxifen resistance. In support of autophagy as a contributor to resistance development, recent studies have demonstrated that autophagy in tamoxifen resistant breast cancer cells promotes survival, which when inhibited induces apoptotic cell death [22, 23, 36]. Furthermore, the ability of autophagy to shelter populations of tumor cells and to allow them to evolve was recently demonstrated in DCIS tissue samples obtained from patients [37]. Cells exhibiting malignant characteristics were able to be cultured from these tissues and form tumors in mice, which were dependent on autophagy.

To better understand the mechanism underlying the induction of autophagy by HDAC inhibition and tamoxifen treatment, we examined the role of the ER. Tamoxifen exerts its

effects by competing as a ligand with estrogen for binding to the ER and perturbing ER-mediated signaling. Consistent with tamoxifen mediating induction through the ER, high dose E2 attenuated autophagy in tamoxifen treated cells. Furthermore, autophagy was inhibited in tamoxifen treated cells when the ER was depleted by siRNA. However, depleting the ER by either siRNA or treatment with the SERD fulvestrant was unable to induce autophagy when combined with an HDAC inhibitor. Whether HDAC inhibitors exert their effect on autophagy induction through ER signaling is less clear. High dose E2 had only a modest effect on induction in cells treated with the combination. Also, in cells depleted by siRNA, the degree to which LC3 was diminished when treated with the combination was significantly less than when solely treated with tamoxifen. Thus, tamoxifen appears to induce autophagy by perturbing downstream ER-signaling, while the HDAC inhibitor likely acts via an alternate pathway. This was consistent with our finding that Beclin-1 was induced differentially with the combination. VPA increased Beclin-1 at the protein level, but not mRNA, while tamoxifen increased both mRNA and protein levels in an ER dependent manner. It is likely that tamoxifen-induced autophagy involves more than Beclin-1 up-regulation, as treatment with VPA alone results in Beclin-1 up-regulation but not LC3. Our finding that only the combination substantially down-regulates Bcl-2 is probably a key factor contributing to the large induction of autophagy compared to treatment with tamoxifen alone.

The anti-tumor activity of tamoxifen is primarily anti-proliferative. However, with the addition of an HDAC inhibitor, cells are directed into apoptosis. Induction of apoptosis appears to be via mitochondrial signaling with increased cytochrome C release. When treated with the combination, apoptosis was associated with an altered balance of apoptotic genes, including the key apoptosis inhibitor Bcl-2 and down-stream activators, Bax and Bak. A study by *Hodges-Gallagher and colleagues* [25] found that treatment with VPA and low dose tamoxifen (10 nM) over long periods (7 days) resulted in up-regulation of Bik. Bik has been shown to antagonize Bcl-2 like apoptotic inhibitors, resulting in the activation of the down-stream apoptotic drivers Bax and Bak. Although under our conditions we were unable to detect changes (data not shown), elevated levels of Bik would support our findings that apoptosis is mediated through the activation of Bax and Bak and release of cytochrome C.

Results from these experiments demonstrate that the addition of an HDAC inhibitor to tamoxifen treatment predominantly redirects ER-positive breast cancer cells into apoptosis. However, autophagy induction in a subset of cells protects them from cell death. Sheltering a population of cells from tamoxifen may be a critical step, providing cells the opportunity to evolve and development tamoxifen resistance (Figure 6). Thus, inhibition of autophagy should be considered with further evaluation of this drug combination, or different HDAC inhibitors might be sought that reduce induction of autophagy, but maintain the promotion of apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

HDAC	histone deacetylase
ER	estrogen receptor
siRNA	small interfering RNA
VPA	valproic acid

References

1. Thomas S, Munster Pn. Histone deacetylase inhibitor induced modulation of anti-estrogen therapy. *Cancer Lett.* 2009; 280(2):184–191. [PubMed: 19185986]
2. Margueron R, Duong V, Castet A, Cavaillès V. Histone deacetylase inhibition and estrogen signalling in human breast cancer cells. *Biochem Pharmacol.* 2004; 68(6):1239–1246. [PubMed: 15313422]
3. Huang, Bh; Laban, M.; Leung, Ch, et al. Inhibition of histone deacetylase 2 increases apoptosis and p21cip1/waf1 expression, independent of histone deacetylase 1. *Cell death and differentiation.* 2005; 12(4):395–404. [PubMed: 15665816]
4. Hrzenjak A, Moinfar F, Kremser M, et al. Valproate inhibition of histone deacetylase 2 affects differentiation and decreases proliferation of endometrial stromal sarcoma cells. *Mol Cancer Ther.* 2006; 5(9):2203–2210. [PubMed: 16985053]
5. Song J, Noh Jh, Lee Jh, et al. Increased expression of histone deacetylase 2 is found in human gastric cancer. *Apmis.* 2005; 113(4):264–268. [PubMed: 15865607]
6. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen Kp, Gottlicher M. Induction of hdac2 expression upon loss of apc in colorectal tumorigenesis. *Cancer Cell.* 2004; 5(5):455–463. [PubMed: 15144953]
7. Halkidou K, Gaughan L, Cook S, Leung Hy, Neal De, Robson Cn. Upregulation and nuclear recruitment of hdac1 in hormone refractory prostate cancer. *Prostate.* 2004; 59(2):177–189. [PubMed: 15042618]
8. Toh Y, Yamamoto M, Endo K, et al. Histone h4 acetylation and histone deacetylase 1 expression in esophageal squamous cell carcinoma. *Oncol Rep.* 2003; 10(2):333–338. [PubMed: 12579268]
9. Osada H, Tatematsu Y, Saito H, Yatabe Y, Mitsudomi T, Takahashi T. Reduced expression of class ii histone deacetylase genes is associated with poor prognosis in lung cancer patients. *Int J Cancer.* 2004; 112(1):26–32. [PubMed: 15305372]
10. Zhang Z, Yamashita H, Toyama T, et al. Quantitation of hdac1 mrna expression in invasive carcinoma of the breast. *Breast Cancer Res Treat.* 2005; 94(1):11–16. [PubMed: 16172792]
11. Krusche, Ca; Wulfing, P.; Kersting, C., et al. Histone deacetylase-1 and -3 protein expression in human breast cancer: A tissue microarray analysis. *Breast Cancer Res Treat.* 2005; 90(1):15–23. [PubMed: 15770522]
12. Duvic M, Talpur R, Ni X, et al. Phase ii trial of oral vorinostat (suberoylanilide hydroxamic acid, saha) for refractory cutaneous t-cell lymphoma (ctcl). *Blood.* 2007; 109(1):31–39. [PubMed: 16960145]
13. Olsen, Ea; Kim, Yh; Kuzel, Tm, et al. Phase iib multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous t-cell lymphoma. *J Clin Oncol.* 2007; 25(21):3109–3115. [PubMed: 17577020]
14. Piekarczyk, Rl; Frye, R.; Turner, M., et al. Phase ii multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous t-cell lymphoma. *J Clin Oncol.* 2009; 27(32):5410–5417. [PubMed: 19826128]
15. Sharma D, Blum J, Yang X, Beaulieu N, Macleod Ar, Davidson Ne. Release of methyl cpG binding proteins and histone deacetylase 1 from the estrogen receptor alpha (er) promoter upon reactivation in er-negative human breast cancer cells. *Mol Endocrinol.* 2005; 19(7):1740–1751. [PubMed: 15746193]
16. Yang X, Phillips D, Ferguson At, Nelson Wg, Herman Jg, Davidson Ne. Synergistic activation of functional estrogen receptor (er)-alpha by DNA methyltransferase and histone deacetylase inhibition in human er-alpha-negative breast cancer cells. *Cancer Res.* 2001; 61(19):7025–7029. [PubMed: 11585728]
17. Fan J, Yin Wj, Lu Js, et al. Er alpha negative breast cancer cells restore response to endocrine therapy by combination treatment with both hdac inhibitor and dnmt inhibitor. *J Cancer Res Clin Oncol.* 2008; 134(8):883–890. [PubMed: 18264725]
18. Bickak E, Marchion Dc, Schmitt M, Munster Pn. Selective inhibition of histone deacetylase 2 silences progesterone receptor mediated signaling. *Cancer Res.* 2008; 68(5):1513–1519. [PubMed: 18316616]

19. Bursch W, Ellinger A, Kienzl H, et al. Active cell death induced by the anti-estrogens tamoxifen and 4-hydroxytamoxifen in human mammary carcinoma cells (mcf-7) in culture: The role of autophagy. *Carcinogenesis*. 1996; 17(8):1595–1607. [PubMed: 8761415]
20. Bilir A, Altinoz Ma, Erkan M, Ozmen V, Aydinler A. Autophagy and nuclear changes in fm3a breast tumor cells after epirubicin, medroxyprogesterone and tamoxifen treatment in vitro. *Pathobiology*. 2001; 69(3):120–126. [PubMed: 11872957]
21. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*. 2004; 23(16):2891–2906. [PubMed: 15077152]
22. Qadir, Ma; Kwok, B.; Dragowska, Wh, et al. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Res Treat*. 2008; 112(3):389–403. [PubMed: 18172760]
23. Samaddar, Js; Gaddy, Vt; Duplantier, J., et al. A role for macroautophagy in protection against 4-hydroxytamoxifen-induced cell death and the development of antiestrogen resistance. *Mol Cancer Ther*. 2008; 7(9):2977–2987. [PubMed: 18790778]
24. Gajdos C, Jordan Vc. Selective estrogen receptor modulators as a new therapeutic drug group: Concept to reality in a decade. *Clin Breast Cancer*. 2002; 2(4):272–281. [PubMed: 11899358]
25. Hodges-Gallagher L, Valentine Cd, Bader Se, Kushner Pj. Inhibition of histone deacetylase enhances the anti-proliferative action of antiestrogens on breast cancer cells and blocks tamoxifen-induced proliferation of uterine cells. *Breast Cancer Res Treat*. 2006
26. Scarlatti F, Bauvy C, Ventruti A, et al. Ceramide-mediated macroautophagy involves inhibition of protein kinase b and up-regulation of beclin 1. *J Biol Chem*. 2004; 279(18):18384–18391. [PubMed: 14970205]
27. Levine B, Sinha S, Kroemer G. Bcl-2 family members: Dual regulators of apoptosis and autophagy. *Autophagy*. 2008; 4(5):600–606. [PubMed: 18497563]
28. Galonek, Hl; Hardwick, Jm. Upgrading the bcl-2 network. *Nat Cell Biol*. 2006; 8(12):1317–1319. [PubMed: 17139279]
29. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. 2007; 87(1):99–163. [PubMed: 17237344]
30. Shintani T, Klionsky Dj. Autophagy in health and disease: A double-edged sword. *Science*. 2004; 306(5698):990–995. [PubMed: 15528435]
31. Aita, Vm; Liang, Xh; Murty, Vv, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics*. 1999; 59(1):59–65. [PubMed: 10395800]
32. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer*. 2007; 7(12):961–967. [PubMed: 17972889]
33. Katayama M, Kawaguchi T, Berger Ms, Pieper Ro. DNA damaging agent-induced autophagy produces a cytoprotective adenosine triphosphate surge in malignant glioma cells. *Cell death and differentiation*. 2007; 14(3):548–558. [PubMed: 16946731]
34. Amaravadi, Rk; Yu, D.; Lum, Jj, et al. Autophagy inhibition enhances therapy-induced apoptosis in a myc-induced model of lymphoma. *The Journal of clinical investigation*. 2007; 117(2):326–336. [PubMed: 17235397]
35. Abedin, Mj; Wang, D.; McDonnell, Ma; Lehmann, U.; Kelekar, A. Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell death and differentiation*. 2007; 14(3): 500–510. [PubMed: 16990848]
36. Schoenlein, Pv; Periyasamy-Thandavan, S.; Samaddar, Js; Jackson, Wh; Barrett, Jt. Autophagy facilitates the progression of eralpha-positive breast cancer cells to antiestrogen resistance. *Autophagy*. 2009; 5(3):400–403. [PubMed: 19221464]
37. Espina V, Mariani Bd, Gallagher Ri, et al. Malignant precursor cells pre-exist in human breast dcis and require autophagy for survival. *PLoS One*. 5(4):e10240. [PubMed: 20421921]

Figure 1.

HDAC inhibitors induce autophagy followed by apoptosis in tamoxifen treated breast cancer cells. **(A)** T47D cells were treated with vehicle, C, 0.5 mM valproic acid, V, 10 μ M 4OH-tamoxifen, T, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen, VT, and western blotted for LC3 and PARP at the indicated times. **(B)** T47D cells were treated for 48 hours with vehicle, C, 1 μ M vorinostat, Vor, 10 μ M 4OH-tamoxifen, T, 1 μ M vorinostat and 10 μ M 4OH-tamoxifen, VorT, 30 nM panobinostat, Pan, or 30 nM panobinostat and 10 μ M 4OH-tamoxifen, PanT, and western blotted for LC3. GAPDH was evaluated as a load control for western blot analysis.

Figure 2.

When treated with an HDAC inhibitor and tamoxifen, distinct populations of cells exhibit induction of autophagy, apoptosis, or the combination. T47D cells were treated with vehicle, 0.5 mM VPA (data not shown), 10 μ M 4OH-tamoxifen, or the combination for the indicated times, stained with anti-cleaved PARP and LC3 antibodies, and analyzed by flow cytometry. Total events captured are presented as dot plots. Cut-off for normal cleaved PARP and LC3 levels were set based on values determined for cells treated with vehicle and presented as the horizontal and vertical lines. Events that lay to the right of the vertical line are considered to have elevated LC3, while events that lay above the horizontal line are considered to have elevated cleaved PARP. The percentages of events that lie within each quadrant are indicated.

Figure 3.

HDAC inhibitors and tamoxifen induce autophagy in part through ER signaling. **(A)** T47D cells grown in an estrogen stripped environment were treated with vehicle, 0.5 mM VPA, 10 μ M 4OH-tamoxifen, Tam, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen, and 0 nM or 1 nM E2 for 48 hours and western blotted for ER α and LC3. **(B)** T47D cells grown in an estrogen stripped environment were treated vehicle, 0.5 mM VPA, 100 nM fulvestrant, Ful, or 0.5 mM VPA and 100 nM fulvestrant, and 0 nM E2 or 1 nM E2 for 48 hours and western blotted for ER α and LC3. **(C)** T47D cells were transfected with scrambled (–) or ER α (+) siRNA. The following day, these cells were treated with vehicle, C, 0.5 mM VPA, V, 10 μ M 4OH-tamoxifen, T, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen, VT, for 48 hours and western blotted for ER α , Beclin-1, and LC3. **(D)** MDA-MB-231 cells were treated with vehicle, C, 0.5 mM VPA, V, 10 μ M 4OH-tamoxifen, T, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen, VT, for 48 hours and western blotted for LC3. GAPDH was evaluated as a load control for western blot analysis.

Figure 4.

Treatment with tamoxifen and an HDAC inhibitor alters the balance of autophagy and apoptosis inhibitors and drivers. T47D cells were treated with vehicle, C, 0.5 mM VPA, V, 10 μ M 4OH-tamoxifen, T, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen, VT, for 48 hours and Beclin-1 protein (A) and RNA (B) was quantified or for 72 hours and western blotted for Bcl-2 (C), Bax (D), and Bak (E). (F) T47D cells were treated with vehicle, C, 0.5 mM VPA, V, 10 μ M 4OH-tamoxifen, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen for 72 hours and the cytoplasm was fractionated and western blotted for cytochrome C. Control T47D cells were treated with 1 μ M of staurosporine (STS) for 24 hours prior to evaluation of cytoplasmic cytochrome C. Relative cytochrome C differences presented are representative of multiple independent experiments. RNA levels were normalized to β -glucuronidase and presented as the average of three independent experiments with the standard error of the mean indicated with error bars. GAPDH was evaluated as a load control for western blot analysis. Western blot quantification for Beclin-1, Bcl-2, Bax, and Bak was conducted using

NIH Image J software. The data was obtained from three independent experiments and presented as the average with the error bars representing the standard error of the mean. Asterisks indicate a p value <0.05 compared to individual treatments.

Figure 5.

Autophagy inhibits the cytotoxicity of VPA and tamoxifen in T47D cells. T47D cells were treated with vehicle, C, 0.5 mM VPA, V, 10 μ M 4OH-tamoxifen, T, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen and with or without 10 μ M chloroquine for 48 hours. Treated cells were western blotted for LC3 (**A**) and assayed for viability by dye exclusion (**B**). (**C and D**) T47D cells were transfected with scrambled or Beclin-1 siRNA. The following day, these cells were treated with vehicle, C, 0.5 mM VPA, 10 μ M 4OH-tamoxifen, T, or 0.5 mM VPA and 10 μ M 4OH-tamoxifen, VT, for 48 hours and viability was assayed by dye exclusion. For viability, each condition was conducted in triplicate and presented as the average with error bars indicating the standard error of the mean. GAPDH was evaluated as a load control for western blot analysis.

Figure 6.

Model illustrating mechanism of tamoxifen and HDAC inhibitor induction of autophagy and apoptosis. When combined, HDAC inhibition and tamoxifen promote both autophagy and apoptosis by altering the balance of inhibitors and drivers of both pathways. Induction of autophagy by HDAC inhibition and tamoxifen shelters a subpopulation of cells from apoptotic cell death, which may provide cells the opportunity to develop tamoxifen resistance. The autophagy-protected population of cells can be redirected to undergo apoptotic cell death by concomitant autophagy inhibition (e.g. Chloroquine (CQ) or Beclin-1 siRNA treatment).