Autophagy Inhibition Augments the Anticancer effects of Epirubicin treatment in Anthracycline-Sensitive and Resistant Triple Negative Breast Cancer

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Translational Relevance

Triple negative breast cancers (TNBCs) are defined by a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (ERBB2/HER2). This subtype of breast cancer is heterogeneous, response to available chemotherapy varies considerably, and most patients with advanced disease eventually develop drug resistance. Herein, we show that epirubicin (EPI) induced autophagy as a pro-survival mechanism in TNBC cells sensitive to anthracyclines and that elevated levels of basal autophagy are present in derivative TNBC cells resistant to anthracyclines. Suppression of autophagy augmented cell death activated by EPI treatment, both in sensitive and resistant TNBC cells in vitro. In TNBC tumor xenograft mouse models, combined treatment of EPI and hydroxychloroquine (HCQ) significantly reduced the growth of both anthracycline sensitive and resistant TNBC tumors, compared to either monotherapy. These data thus provide a rationale for clinical evaluation of HCQ in combination with anthracycline therapy in advanced TNBC.
Abstract:

**Purpose:** Triple negative breast cancers (TNBCs) are defined by a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (ERBB2/HER2). Although initially responsive to chemotherapy, most recurrent TNBCs develop resistance, resulting in disease progression. Autophagy is a lysosome-mediated degradation and recycling process that can function as an adaptive survival response during chemotherapy and contribute to chemoresistance. Our goal was to determine whether autophagy inhibition improves treatment efficacy in TNBC cells in tumors either sensitive or refractory to anthracyclines.

**Experimental Design:** We employed *in vitro* and *in vivo* models of TNBC using cell lines sensitive to epirubicin (EPI) and other anthracyclines, as well as derivative lines, resistant to the same drugs. We assessed basal autophagy levels and the effects of chemotherapy on autophagy in parental and resistant cells. Applying various approaches to inhibit autophagy alone and in combination with chemotherapy, we assessed the effects on cell viability *in vitro* and tumor growth rates *in vivo*.

**Results:** We demonstrated that EPI induced autophagic flux in TNBC cells. EPI-resistant lines exhibited at least 1.5 fold increased basal autophagy levels and, when treated with autophagy inhibitors, showed a significant loss in viability, indicating dependence of resistant cells on autophagy for survival. Combination of EPI with the autophagy inhibitor hydroxychloroquine (HCQ) resulted in a significant reduction in tumor growth compared to monotherapy with EPI.

**Conclusion:** Autophagy inhibition enhances therapeutic response in both anthracycline-sensitive and resistant TNBC and may be an effective new treatment strategy for this disease.
Introduction:

The term “triple-negative” breast cancer (TNBC) was coined in 2005 (1) for cancers lacking detectable expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/NEU/ERBB2). TNBC accounts for 10-15% of all breast cancers and although the majority of tumors are basal-like cancers, other TNBCs exist and are described by both genomic and histopathological features (2). Due to the lack of hormone receptors and HER2 expression, current treatment strategies are limited to chemotherapeutic agents. Various targeted agents such as anti-EGFR drugs have been studied but with limited success (2). In the advanced setting, TNBC patients have poorer outcomes due to drug resistance and more aggressive recurrent disease compared to other subtypes (2). Recent gene expression (3) and RNA sequencing analyses (4) showed that TNBCs are particularly diverse in their molecular expression profiles, and in the mutation status of oncogenes and tumor suppressor genes, respectively. Due to this heterogeneity, TNBCs may respond differently to available treatment strategies, presenting a further clinical challenge. Alternative treatment strategies are required to improve outcomes for patients with TNBC.

Macroautophagy, hereafter termed autophagy, is an intracellular process that involves formation of double membrane structures known as autophagosomes that sequester cytoplasmic material. Autophagosome maturation involves fusion with lysosomes to form autolysosomes, where hydrolysis of the contents occurs (5–7). The hydrolyzed components are then released back into the cytoplasm and can be used to generate new proteins and lipids in addition to energy required for cellular biosynthesis during nutrient deprivation or metabolic stress (6–9). Autophagy occurs at basal levels to degrade long-lived cytosolic proteins and organelles but is...
also upregulated to promote cell survival in response to nutrient starvation (10) and other cellular stresses including radiation (11), chemotherapy (12) and oxidative stress (13).

Autophagy has been implicated in multiple human diseases, including cancer progression and in the cellular response to cancer treatments (5,6). Anti-cancer therapies commonly lead to the upregulation of autophagy (14) and in this context, autophagy has a cytoprotective function that enables cancer cells to cope with cytotoxic or other stresses induced by treatment (15,16). The cytoprotective and/or survival functions of autophagy provide an opportunity for combinatorial therapy, whereby autophagy inhibition can be applied concurrently with anti-cancer drugs or radiation therapy to increase treatment efficacy (5,6,11,12,17–22). Studies have employed either genetic strategies to inhibit essential autophagy proteins, or pharmacological strategies that utilize late-stage autophagy inhibitors such as bafilomycin A1 (BAF), chloroquine (CQ) or hydroxychloroquine (HCQ). These agents act by increasing lysosomal pH, thereby impairing lysosomal degradation, and often leading to an accumulation of autophagosomes due to a block in their fusion with lysosomes (23). Several phase I/II clinical studies involving autophagy modulation using FDA-approved CQ or HCQ in combination with chemotherapy or radiation for the treatment of different cancers, including breast, colon, glioma, prostate and non-small cell lung cancers, are currently underway (5,24).

Chemotherapy resistance remains a major hurdle in the treatment of advanced TNBC and increasing evidence indicates that autophagy may contribute to the development of treatment resistance (16,25,26). In this study, we show that epirubicin induced autophagic flux in TNBC cell lines. We developed anthracycline-resistant TNBC cell lines and demonstrate that in the absence of treatment they exhibit relatively high levels of autophagic flux, compared to their parental cell lines. We provide proof-of-principle evidence supporting the effective combination
of EPI with pharmacological (CQ; HCQ) or genetic (siRNA) inhibition of autophagy, both in TNBC parental cell lines and their derivative anthracycline-resistant lines, \textit{in vitro} and in xenograft mouse models. Our findings indicate that combinatorial autophagy inhibition may be part of an effective treatment option for both primary and recurrent anthracycline-resistant TNBCs.

\textbf{Materials and Methods:}

\textbf{Reagents:}

The following antibodies were used: anti-LC3B (Abcam); anti-b-Actin (Abcam); anti-ATG7 (Novus); anti-ATG5 (Cell Signaling); anti-p62 (BD Biosciences); anti-PERK (EIF2AK3) (Cell Signaling); anti-BiP (HSPA5) (Cell Signaling) anti-PARP (Cell Signaling); IRDye\textsuperscript{TM}700DX conjugated anti-rabbit IgG and IRDye\textsuperscript{TM}800DX conjugated anti-mouse IgG (Rockland); goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Santa Cruz). The following drugs were used: hydroxychloroquine (HCQ; Acros Organics); chloroquine (CQ; Sigma-Aldrich) and bafilomycin A1 (BAF) (Sigma-Aldrich); epirubicin (EPI; Pharmaceutical Partners of Canada INC.); doxorubicin (Pfizer Canada Inc); mitoxantrone (Hospira Healthcare Corporation).

\textbf{Cell lines and reagents}

For \textit{in vitro} experiments, MDA-MB-231 cells (2005; ATCC-authentication by isoenzyme and STR analyses) were maintained at 37\textdegree C, 5\% CO2 in Gibco DMEM (Life Technologies) supplemented with 10\% fetal bovine serum (FBS). For \textit{in vivo} experiments, MDA-MB-231 cells were grown in Gibco RPMI 1640 medium with 10\% FBS. SUM159PT cells (2011, Asterand-authentication by STR analysis) were maintained at 37\textdegree C, 5\% CO2 in Ham's F-12 medium with 10\% FBS, HEPES (Sigma-Aldrich), 5 \mu g/ml insulin (Sigma-Aldrich), and 1 \mu g/ml
hydrocortisone (Sigma-Aldrich). BT549 cells (2011, ATCC--authentication by isoenzyme and STR analyses) were maintained at 37°C, 5% CO2 in Gibco DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 5µg/ml insulin (Sigma-Aldrich).

Resistant cell lines MDA-MB-231-R8 and SUM159PT-R75 were generated by growing in increasing concentrations (12.5-100nM for R8; 100nM-1µM for R75) of EPI for approximately one year and 6 months, respectively, and were maintained in culture with 100 nM and 1µM EPI, respectively. Prior to any experiments, the cells were withdrawn from EPI for at least 2 passages.

Cell viability assays

In all short-term experiments, except for the IC50 assay, cell viability was determined by the trypan blue exclusion assay, using a Countess® Automated Cell Counter (Life Technologies). To establish IC50, Alamar Blue–based metabolic assay (Invitrogen) was used. Data for IC50 evaluations are presented as percentages relative to the untreated control (means ± SEMs) and calculated using non-linear regression analysis, Prism 6.0 (GraphPad Software Inc.).

Autophagic flux assay with mRFP-GFP-LC3B reporter:

For autophagic flux experiments with mRFP-GFP-LC3B (see supplementary methods), 2 x 10⁴ cells were plated for 24 hrs in Lab-TekII Chamber Slides (Nalge Nunc). MDA-MB-231 and SUM159PT cells were treated with 100nM and 200nM EPI respectively for 48 hours. To inhibit flux, 15nM and 25nM BAF (for MDA-MB-231 and SUM159PT, respectively) was added 5hrs before fixing the cells with 4% paraformaldehyde. Cells were mounted with Slowfade Gold Reagent (Invitrogen) and were imaged with a Nikon Confocal C1 microscope equipped with a
Plan APO 60X/1.45 oil immersion objective (Nikon). Images were acquired using EZ-C1 Version 3.00 software (Nikon), and brightness and contrast were adjusted with Photoshop (CS4, Adobe) and applied to the whole image. For quantitations of puncta, images were captured with a Zeiss Axioplan 2 microscope. Images were acquired with Axio Vision release 4.8.2.0 software (Carl Zeiss) at red and green channels. For each treatment, puncta from red channel minus green channel (net red puncta) was determined for 100 cells manually.

**Clonogenic and crystal violet proliferation recovery assay (hereafter referred to as clonogenic crystal violet assay)**

Cells were plated at 1x10^3 cells per well in a 6-well plate. After 24 hrs, drugs were added and cells incubated for 4 days. Media with drug was replaced with fresh media without drug and cultured for another 6-7 days. Colonies were fixed with 4% PFA and stained with 10% crystal violet. Images of colonies were captured with a LAS4000 imaging system (Fuji). Retained crystal violet stain was resolubilized in 10% acetic acid by rocking for 15 min. Readings were taken at A_{590} using a plate reader (VersaMAX Microplate Reader).

**siRNA transfection and Epirubicin treatment**

Stealth siRNA from Invitrogen was used for all the siRNA experiments. For combination treatment of siRNA and EPI, cells were plated at 2x10^5/well (6-well plates) in serum-free medium and transfected with 75 pmol siRNAs or a scramble siRNA control. The day after transfection, the serum-free medium was replaced with fresh media containing 10% FBS, and cells were treated with 25nM epirubicin. Twenty-four hours post-treatment, cell viability and western blot assays were performed.
For treatments with siRNA only, cells were transfected under serum free conditions followed by the addition of serum (10% FBS, final) after 16hrs. 40 hrs after the initial siRNA transfection, cells were transfected again with respective siRNAs under serum-free conditions. Media with serum was added after the second transfection and cells were harvested 112hrs from the time of the first siRNA transfection.

**Western blot**

Protein lysates were prepared using RIPA Lysis Buffer Kit (Santa-Cruz), according to the manufacturer’s protocol. Electrophoresis and transfer of proteins were performed using standard methods and protein–antibody complexes were detected by either Infrared or chemiluminescence assays. Quantitation of the signal was performed using ImageQant 5.1 software or Bio-Rad image analysis software. Autophagic flux assay was employed using lysosomal inhibitor Bafilomycin (BAF) and western blot analysis (27) (details in supplementary methods).

**Caspase-Glo 3/7 assay (Promega)**

8x10^3 MDA-MB231-R8 or SUM159PT-R75 cells were plated in each well of 96-well optical plates (BD Biosciences) and transfected with scramble or ATG-siRNAs. Staurosporine or cycloheximide served as controls. Ninety hours after the first transfections, caspase levels were determined with Caspase-Glo® 3/7 kit. Luminescence was measured with a Synergy H4 Hybrid (Biotek).

**Animal studies**

Animal protocols were reviewed and approved by the Institutional Animal Care Committee (IACC) at the University of British Columbia prior to conducting experiments. The care, housing
and use of animals were performed in accordance with the Canadian Council on Animal Care Guidelines.

Female Rag2M mice (n=6 to 10 per treatment group) were used to assess tumor growth and autophagy markers. MDA-MB-231 or MDA-MB-231-R8 cells (1×10^7 in matrigel) were injected subcutaneously in 9-14-week-old mice, and tumor growth was monitored. Once the average tumor volume reached approximately 100 mm^3, mice were randomized into treatment groups. To evaluate the immediate effects of HCQ on autophagy levels in tumors, a bolus of HCQ at a relatively high dose, 120 mg/kg, was administered by intra-peritoneal injection in tumor-bearing mice. After 2, 4, and 24 hours post-injection the tumors were harvested and analyzed for LC3B-II and p62 levels. To determine the therapeutic efficacy of EPI, HCQ, or their combination, mice were treated for up to 4 weeks with saline, maximum tolerated dose (MTD) of EPI (I.V. 7mg/kg, weekly), HCQ (I.P. 90mg/kg, daily ×5 days/week), or both. Tumor size measurements with calipers as well as monitoring for signs of toxicity (e.g. bodyweight loss, behavioral changes) were performed twice a week and daily, respectively. Four hours after the last injection of HCQ animals were terminated, and a necropsy was performed to evaluate drug toxicity. Each harvested tumor was used for western blot assay of LC3B-II and p62 levels and immunohistochemical (IHC) analysis (see below).

**Hematoxylin and eosin (H&E) and IHC Staining**

Tumor samples for IHC were fixed in 10% neutral buffered formalin, transferred to 70% ethanol after 24 hours, and stored at 4°C until processed. H&E staining was essentially performed as described in Wang et. al. 2005 (28) and for IHC the LC3B antibody from abcam (cat# ab48394) was stained on the Ventana DiscoveryXT (details in Supplementary).
Statistical Analyses

For *in vitro* cell viability or proliferation assays comparing combination treatment to EPI treatment alone, Student’s t test (two-tailed, unpaired) was employed. For multiple comparisons (caspase assay) one-way ANOVA with Dunnett’s post-test was used.

Statistical significance of differences in tumor measurements between groups was calculated using the Kruskal-Wallis test with Dunn’s correction for multiple comparisons. For survival analysis, a Log-rank (Mantel-Cox) test was applied. Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc.).

Results:

Epirubicin treatment reduces viability and increases autophagic flux in TNBC cells.

To determine the effects of EPI on TNBC cells, MDA-MB-231 and SUM159PT cells were treated with EPI at varying concentrations. EPI treatment (≥100nM) affected the morphology of MDA-MB-231 cells after 48hrs; cells appeared increased in size with enlarged nuclei (Fig. 1A). At 400nM EPI, a reduction in cell numbers was clearly observed under the inverted microscope (Fig. 1A). Cell counts by the trypan blue cell exclusion assay following 48hrs of 100nM and 400nM EPI treatment showed 46+/-9.6% and 17+/-2.9% viable cells, respectively, compared to the no treatment control (Fig. 1A).

To determine whether EPI treatment increases autophagic flux in TNBC cells, we conducted analyses employing autophagy protein MAP1LC3B (LC3B) and the lysosomal inhibitor BAF (details in methods) at its established saturating concentrations (Supplementary Fig. S1). As indicated by increased levels of LC3B-II in the presence of BAF, and as shown in Fig. 1B in MDA-MB-231 and SUM159PT cells, increased autophagic flux was observed from 6hrs and up
to 6 days after drug treatments. These results show that EPI treatment induces autophagic flux, and that high levels of autophagy are maintained in the remaining cells that survive EPI treatment. Consistent with this finding, we observed that EPI treatment also leads to increased transcript levels of autophagy genes in TNBC cells that survived EPI treatment (Supplementary Fig. S2A and S2B).

To confirm our results, we used an alternate flux assay employing a mRFP-GFP-LC3B construct stably integrated in MDA-MB-231 and SUM159PT cells (see (28) and methods). As shown in Fig. 1C, both MDA-MB-231 and SUM159PT cells treated with EPI showed increased RFP positive red puncta compared to untreated cells (p<0.001), indicating that EPI treatment induced autophagic flux. Control cells treated with BAF showed an increase in yellow puncta indicating an increase in autophagosomes but an obstruction of autophagic flux. Together with the BAF/LC3B-II western blot assay, these results show that EPI induces autophagic flux in TNBC cells. To identify potential molecular mechanisms related to EPI treatment effects, we determined the levels of ER stress proteins HSPA5 (GRP78/Bip) and EIF2AK3 (PERK) at 6 hrs and 6 days after EPI treatment in MDA-MB-231 cells (Fig. 1D). Both proteins showed elevated levels following EPI treatment, which also correlated with the increased autophagic flux.

**TNBC cells resistant to Anthracyclines show increased basal autophagy.**

To evaluate the role of autophagy with respect to anthracycline resistance, we derived TNBC polyclonal lines resistant to EPI. Compared with the parental MDA-MB-231 EPI-sensitive line, the derivative MDA-MB-231-R8-resistant line (hereafter referred to as R8) had an 8 fold higher IC$_{50}$ (MDA-MB-231 IC$_{50}$ (95% confidence interval (CI)) = 515 nM (389-683 nM) versus R8 IC$_{50}$ (95% CI) = 4272 nM (3155-5783 nM); P≤0.0001; Fig. 2A). Similarly, the derivative SUM159PT-resistant cell line (SUM159PT-R75, hereafter referred to as R75) had a 147 fold
higher IC₅₀ compared to the sensitive parental line (SUM159PT IC₅₀ (95% CI) = 56 nM (35-90 nM) versus R75 IC₅₀ (95% CI) = 8251 nM (5954-11434 nM; P ≤ 0.0001; Fig. 2A). The parental line SUM159PT appears to be more sensitive to EPI compared to MDA-MB-231 however, the derived R75-resistant line was more resistant to EPI compared to the R8-resistant line. As shown in Supplementary Table S1, R8 and R75 lines also demonstrated resistance to other anthracyclines (doxorubicin and mitoxantrone). The MDA-MB-231-derived resistant line R8 showed 80+/-3% and 46+/-6% viable cells with 100nM and 200nM EPI treatment, respectively, compared to their parental cells, which showed <15% viable cells at both concentrations 7 days after treatment (Fig. 2B). These results show that R8 and R75 are anthracycline-resistant cell lines.

To determine whether autophagy is altered in the resistant lines R8 and R75, we compared the basal levels of autophagic flux in resistant and parental lines at saturating concentrations of BAF (Supplementary Fig. S1; 10 and 40 nM for R8 and R75 respectively). Both resistant lines clearly showed an elevated level of autophagic flux relative to their respective parental lines (Fig. 2C). To confirm this observation, we stably transfected R8 and R75 with the reporter mRFP-GFP-LC3B. Both of these EPI-resistant lines showed higher levels of red puncta compared to their respective parental lines (Fig. 2D), indicating an increase in autophagic flux. These experiments show that the anthracycline resistant lines R8 and R75 have elevated levels of basal autophagy compared to their respective parental lines.

**Chemotherapy in combination with autophagy inhibition is more effective than monotherapy in reducing viability of both EPI-sensitive and EPI-resistant TNBC cells in vitro.**
To determine whether autophagy inhibition sensitizes cells to EPI, we specifically inhibited autophagy with siRNAs targeting essential autophagy-related (ATG) proteins ATG7 or ATG5 in combination with a low concentration of EPI. Knockdown of ATG targets was confirmed using western blot analysis (Fig. 3A). The effects of ATG knockdown with and without EPI on cell viability were assessed by trypan blue assays. ATG7-siRNA or ATG5-siRNA combined with EPI treatment reduced the viability of MDA-MB-231 by approximately 20% and 30% respectively (p≤0.0001 for ATG7+EPI and p≤0.001 for ATG5-siRNA+ EPI) compared to MDA-MB-231 cells treated with EPI and control scramble siRNA (Fig. 3B). As shown in Fig. 3B, combined treatment of autophagy inhibition with ATG7-siRNA or ATG5-siRNA and EPI treatment reduced the viability of SUM159PT cells by 20% and 12% respectively (p≤ 0.00003 for ATG7+EPI and p≤0.009 for ATG5-siRNA+ EPI) compared to controls. These results suggest that in TNBC cells, the knockdown of ATG7 or ATG5 further decreases cell viability upon EPI treatment.

Since pharmacological inhibition of autophagy with CQ or HCQ is currently being tested in clinical trials, we evaluated the combination effects of CQ and EPI on cell recovery and proliferation using MDA-MB-231 cells. We performed clonogenic crystal violet recovery assays where cells were treated with sub-lethal concentrations of EPI (12.5nM) and CQ (3 or 6uM), either alone or in combination. The clonogenic crystal violet assay showed 36+/−25% proliferating cells for CQ (6uM) and 60+/−17% proliferating cells for EPI treatments compared to untreated cells. Combination of 6µM CQ and 12.5nM EPI was most effective in reducing proliferating cells with 9.6 +/−8% remaining cells compared to no treatment (Fig. 3C). The effect of EPI and CQ combination treatment was also determined in the TNBC cell line BT549, which showed a 43+/−11% reduction in viability compared to EPI treatment alone (Supplementary Fig.
S3). To determine whether other anthracyclines in combination with CQ may effectively reduce clonogenicity of TNBC cells compared to anthracycline treatment alone, we treated MDA-MB-231 cells with Doxorubicin (DOX) alone, CQ alone or in combination and performed crystal violet recovery assays. Our data showed that combination of DOX+CQ was most effective in reducing clonogenicity with 33+/−6% proliferating cells compared to 69+/−6% and 66+/−23% proliferating cells for DOX or CQ alone, respectively (Fig. 3C). These results indicate that anthracyclines or CQ treatments alone can significantly reduce clonogenicity of MDA-MB-231 cells but that combination of anthracyclines and CQ can more effectively eliminate cells. These results taken together indicate that either genetic or pharmacological inhibition of autophagy combined with anthracyclines can be more effective in reducing viability and recovery of anthracycline-sensitive TNBC cells than treatment with autophagy inhibition or anthracycline alone.

To determine whether combination treatment with EPI and CQ is also more effective compared to EPI alone in EPI-resistant cells, the resistant cell lines were treated with sub-lethal concentrations of individual drugs or their combination, and cell viability was determined by the trypan blue assay. In R8, the largest reduction in viable cells (37+/−8%; $p≤0.02$) was observed with combined treatment of CQ and EPI (Fig. 4A) compared to EPI treatment alone. Similar results were obtained with R75 cells, where the combined treatment showed 22%/−11% ($p≤0.025$) reduction in viability compared to EPI alone (Supplementary Fig. S3B). Cells treated with CQ+EPI showed high levels of accumulated LC3B-II protein indicating autophagic flux is inhibited in these cells and may at least in part contribute to the observed reduction in viability (Fig. 4A).
To determine clonogenicity after drug treatments, we treated R8 cells with CQ, EPI or CQ + EPI and performed the crystal violet recovery assay. As expected, the resistant R8 cells treated with EPI alone recovered and produced large clones. The largest reduction in clonogenicity was observed with CQ+EPI treatment (Fig. 4B). The proliferation recovery assay (Fig. 4B) showed significant reduction in proliferating cells for CQ+EPI treatment compared to EPI treatment alone (p≤0.02).

To determine whether specific autophagy inhibition reduces the viability of EPI-resistant TNBC lines R8 and R75, we used siRNAs to knock down autophagy gene products ATG5 or ATG7. Either siRNA treatment resulted in a dramatic reduction in viability of resistant cells R8 and R75 compared to scramble siRNA controls by 7 days (Fig. 4C). To confirm that the ATG5 and ATG7-siRNA treatments disrupt autophagy, we performed autophagy flux assays with saturating concentrations of BAF (Supplementary Fig. S1). As shown for R8 cells (Fig. 4C), compared to scramble controls, cells treated with ATG5-siRNA or ATG7-siRNA led to reduced LC3B-II levels with or without BAF indicating that autophagic flux is significantly reduced. These results show that inhibition of autophagy alone leads to reduction in viability in anthracycline-resistant TNBC cells.

To determine whether autophagy inhibition triggers caspase-dependent cell death, we treated R8 and R75 cells with ATG5-siRNA or ATG7-siRNA and determined the activity levels of caspases 3 and 7. Staurosporine and cycloheximide treatments served as positive controls for caspase activity. Results in Fig. 4D show that ATG5-siRNA or ATG7-siRNA treatment leads to increased levels of caspase activity compared to the scramble-siRNA control (p < 0.0001 in R8 and p < 0.05 in R75). To confirm the increase in caspase activity in R8 and R75 cells treated with ATG-siRNA, we determined the levels of PARP (a substrate for effector caspases 3/7)
using western blot analysis. R8 and R75 cells treated with either ATG5-siRNA or ATG7-siRNA showed an increase in cleaved PARP (Fig. 4D) further supporting that that inhibition of autophagy contributes to caspase-dependent cell death in EPI-resistant TNBC cells.

These assays taken together confirm, in vitro, that combination of autophagy inhibition and EPI is more effective compared to either treatment alone in both EPI-sensitive and EPI-resistant cells. These findings also show that autophagy inhibition contributes, at least in part, to caspase-dependent cell death in EPI-resistant TNBC cells in vitro.

**HCQ inhibits autophagy and sensitizes MDA-MB-231 and R8 tumors to epirubicin treatment**

To confirm that HCQ inhibits autophagy in vivo, Rag2M mice with established MDA-MB-231 xenograft tumors were given a bolus high dose of HCQ (120mg/kg). Accumulation of LC3B-II in HCQ-treated tumors peaked at 4 hours (Fig. 5A). The increased levels of LC3B-II may indicate autophagy inhibition in vivo. Treatment with HCQ and EPI would be predicted to increase LC3B-II levels because of autophagy induction by EPI and late-stage autophagy inhibition by HCQ. IHC analysis of tumor samples stained for LC3B (Fig. 5B) shows elevated levels of LC3B in all treatment groups compared to controls, supporting this prediction.

The in vivo efficacy of EPI and HCQ alone and in combination was assessed at their MTDs (7mg/kg, and 90mg/kg, respectively). MDA-MB-231 tumor growth in mice treated with EPI or HCQ was moderately reduced compared to saline (p=0.0025 and p=0.017, respectively), however, the tumors continued to increase in size. In contrast, tumor growth in mice treated with the combination of EPI and HCQ was significantly reduced compared to saline (by more than 50%, p<0.0001) or monotherapy with EPI (by more than 20%, p<0.01) and the tumors remained small without an increase in size (Fig. 6A) for a time period until saline treated animals needed
to be euthanized for humane reasons. To determine tumor median growth time (MGT), Kaplan-Meier plots for each treatment group were generated (Fig. 6A). The survival endpoint used for these data was defined as the percentage of animals with less than 50% increase in tumor volume (death as an experimental endpoint is not accepted by the IACC). MGT was 7, 15, and 18 days for saline controls, EPI alone, and HCQ alone, respectively; none of the HCQ+EPI treated tumors reached 1.5x original size during the treatment period. Log-rank (Mantel-Cox) test indicated that the combination treatment resulted in a significant increase in MGT compared to saline controls ($p < 0.0001$) and EPI alone ($p < 0.05$). HCQ treatment alone significantly increased MGT compared to saline controls ($p < 0.005$) and had a similar effect ($p=0.95$) to EPI alone.

The toxicity of the treatment regimens described above was mild. Less than 10 percent body weight loss was noted in all the groups of mice. No animals required discontinuation of the treatment and the toxicity data collected suggested that HCQ/EPI combination can be given safely at a dose that augments anti-tumor activity of EPI in EPI-sensitive TNBC.

To follow up on our in vitro results that suggested that anthracycline-resistant cancer cells could be sensitized to chemotherapy by autophagy inhibition, we treated mice bearing MDA-MB-231-R8 tumors with EPI, HCQ or their combination. Therapy with either HCQ or EPI had minimal or no effect on MGT when used as single agents (Fig. 6B). However, combinatorial treatment with EPI and HCQ significantly reduced tumor growth compared to saline controls ($p<0.05$). MGT was 13 days for the saline and EPI alone groups, 16 days for the HCQ alone group, and 24 days for the EPI+HCQ group ($p<0.01$, log-rank test). Half of the mice (7 out of 14) in the combination therapy group had less than a 50% increase in tumor volume by the end of the study. Less than 5% bodyweight loss was noted in all treatment groups. These results
indicate that HCQ sensitizes anthracycline-resistant tumors to EPI, and the combination therapy with HCQ and EPI is an effective treatment. The results of IHC analysis of both parental and resistant tumors from the control groups are shown in Fig. 6C. LC3B staining revealed a substantial difference in LC3B accumulation between parental and resistant tumors, suggesting that higher autophagy levels in resistant tumors, previously seen in our \textit{in vitro} experiments, might partially explain the differences in the effectiveness of combination therapy.

**Discussion:**

TNBC is a particularly aggressive form of breast cancer with limited treatment options due to the lack of targeted therapies. The effectiveness of autophagy modulation alone or in combination with DNA damaging agents in TNBC cells is still an unknown. EPI is a structural analog of doxorubicin that is commonly used to treat TNBC. EPI, however, is usually better tolerated (29). To our knowledge, this is the first report to demonstrate the effectiveness of autophagy inhibition in combination with EPI in both anthracycline-sensitive and -resistant TNBC cells \textit{in vitro} and \textit{in vivo}.

It is demonstrated that treatment of EPI sensitive TNBC cells with EPI (100-200nM) induces autophagic flux that functions to promote cell survival. A previous study showed that high-dose EPI treatment (2.5\(\mu\)M) induced autophagy as a protective mechanism in ER+ MCF7 breast cancer cells (30). In agreement with that study, our analyses showed that autophagy inhibition using RNAi methods led to reduced survival of TNBC cells. We further evaluated autophagy inhibition \textit{in vivo} and observed a significant reduction in xenograft tumor growth when we combined HCQ (90mg/kg) with 7.0mg/kg (weekly) of epirubicin (see Fig. 6). In humans, several phase II studies have used epirubicin as a single agent at doses between 12mg/m\(^2\) – 30 mg/m\(^2\) (weekly) to treat advanced breast cancer (31) and higher doses of 90mg/m\(^2\) have also been
reported (32). Using the FDA guidelines for converting animal doses to human doses, the 7.0mg/kg EPI in mice converts to 20mg/m² in humans and therefore represents a clinically relevant range (33). In another study involving locally advanced primary breast cancer patients, four cycles of epirubicin at 90mg/m² given every three weeks were administered and pharmacokinetics assessed. The maximal mean levels of epirubicin in circulating plasma, tumor tissue and subcutaneous adipose tissue were determined to be 1264ng/ml, 13.3ng/ml and 11.2ng/ml, respectively (32). Circulating plasma levels declined rapidly to less than 100ng/ml by 2 hrs after epirubicin administration. The concentrations of epirubicin used in our in vitro studies were typically in the range of 12.5nM to 200nM, equivalent to 7.3ng/ml to 116ng/ml epirubicin and thus were also in a relevant range.

Our findings suggest that the induction of autophagy in response to epirubicin may also contribute to the development and/or maintenance of epirubicin resistance. The EPI-resistant sub-lines, derived from sensitive TNBC lines, had relatively high levels of basal autophagic flux (Fig. 2C and 2D). It is still formally possible that the high levels of red puncta observed in the resistant lines (Fig. 2D) could indicate a blockade of flux (i.e. at the lysosomal degradation step so that RFP would not be degraded). However, we think this is an unlikely possibility given that a block in autophagy results in a significant reduction in viability and increased apoptosis in these lines (Fig. 4), and we do not observe this under standard culture conditions. An increase in basal autophagy in chemotherapy-resistant lines compared to their sensitive parental lines has been reported previously in breast cancer cell lines (12,34,35) and other cancer (36) cell lines. Our IHC studies of parental and R8 line xenograft tumors (see Fig. 6C), showed elevated levels of LC3B protein in R8 tumors compared to the MDA-MB-231 parental tumors suggesting resistant tumors also maintain higher levels of autophagy in vivo even when the selective
pressure (EPI) is not present. Recent IHC studies have described an association between elevated expression of autophagy proteins LC3A, LC3B and Beclin 1 in TNBC tumors compared to other molecular subtypes of breast cancer (37) and high LC3B was associated with progression and poor outcome in TNBC (38). Understanding the functional significance of elevated autophagy in different breast cancer cell types, along with the potential role and molecular mechanisms of autophagy in drug resistance will be important areas for future study.

Autophagy inhibition in combination with pharmacological aggravators of endoplasmic reticulum stress in TNBC (19,39) and in combination with various drugs in other types of breast cancer cells (12,30,34,40), is known to enhance cell death. These findings are consistent with our results where caspase activation and increased PARP cleavage was clearly observed in EPI-resistant R8 and R75 cells following knockdown of autophagy with ATG-siRNAs (see Fig. 4). In the sensitive cells, we found that EPI treatment led to increased levels of ER stress proteins (Figure 1D). Since ER stress is a known inducer of autophagy and cell death (41), it is possible that the observed effects of EPI may be mediated at least in part through this stress pathway.

It is noteworthy that we and others (5) have observed considerable variability in the cellular response to ATG-siRNAs, BAF, CQ or HCQ. This variability included the saturating concentrations required for autophagy inhibition and concentrations required to alter cancer cell viability. Both MDA-MB-231 and SUM159PT cell lines harbor mutations in P53 and Ras, and evidence indicates that alterations in these proteins may affect dependency on the autophagy process for survival and influence sensitivity to HCQ (42–45). HCQ may also exert additional effects such as immunomodulation and increased drug cytotoxicity due to changes in lysosomal pH (46,47). While HCQ (i.e. a CQ derivative) may alter processes other than autophagy, our in vitro studies employing either CQ or ATG-siRNAs clearly show that autophagic function is
impaired following treatment of cells with these agents (see Fig. 4). CQ had similar effects to ATG5 or ATG7-siRNA in cells in vitro, suggesting that autophagy inhibition is at least in part responsible. Additional studies investigating genetic backgrounds, along with the ongoing clinical trials will help determine the underlying factors and extent of differential responses to HCQ in cancer patients.

While our results showed that combination therapy, relative to monotherapy of either HCQ or EPI, was most effective in both sensitive (MDA-MB-231) and resistant (R8) TNBC cell lines and xenograft models, the growth of sensitive tumors was suppressed more effectively than the growth of resistant tumors (compare results in Fig. 6A and Fig. 6B). It is likely that other mechanisms, in addition to autophagy, contribute to treatment resistance in the R8 tumors. The R8 cells grew more slowly as a tumor than the parental MDA-MB-231 cells (saline controls in Fig. 6B vs 6A) and this could also help explain the reduced EPI activity in R8 tumors. We did not detect a difference in growth rate between the parental and R8 cells in vitro, but this may be a reflection of the longer time frame under which the cells were followed in vivo. Another possible explanation for the difference in treatment response between sensitive versus resistant tumors might be the higher levels of autophagy in the resistant cells, which would then require more potent autophagy inhibition compared to parental cells. In our studies HCQ was already administered at its MTD. It is possible that more potent lysosomal inhibitors, such as Lys05 (48), or small molecule inhibitors targeting essential autophagy proteins could be tested in the future. Further, there may be a role for drug carrier technology here, where nanoscale delivery systems could be used to ensure that drugs such as HCQ could be maintained at high levels over extended time periods. Collectively, our findings demonstrate that autophagy inhibition in combination with epirubicin has the potential to be effective in reducing cell viability and suppressing TNBC.
tumor growth. These data provide a foundational rationale for clinical trials of such a strategy in patients with either primary or recurrent TNBC.

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References:


Figure legends:

Figure 1. Epirubicin (EPI) treatment reduces cell viability and increases autophagic flux in TNBC cells:

A. EPI treatment reduces viability of MDA-MB-231 cells. Cells were visualized with an inverted microscope following treatments with the indicated concentrations of EPI for 48 hrs. Arrowheads indicate large nuclei in treated cells. Trypan blue cell exclusion assay was used to measure the % viable cells following 100, 200, and 400 nM EPI treatments. The percent of viable cells was calculated relative to the untreated control. Error bars represent SD from 3 independent experiments.

B. EPI treatment induces autophagic flux in MDA-MB-231 and SUM159PT TNBC cells. MDA-MB-231 cells were treated with 100 nM EPI for 6 hrs or 6 days. For flux assays, cells were treated with 10 nM Bafilomycin (BAF) alone or 10 nM BAF plus 100 nM EPI. SUM159PT cells were treated with 400 nM EPI for 24 hrs or 5 days. For flux assays, cells were treated with 20 nM BAF alone or 20 nM BAF plus 400 nM EPI. Cells were harvested at the indicated time points, and LC3B-II and Actin levels were evaluated by immunoblot analysis. Intensity of LC3B-II and Actin levels were determined using densitometry analysis. Bar graphs shown represent normalized intensity levels of LC3B-II (LC3B-II/Actin) relative to no treatment controls, with SD from 3 biological replicates. P-values represent the comparisons between BAF alone and BAF+EPI (flux) using Student’s t-test.

C. MDA-MB-231 and SUM159PT cell lines stably expressing mRFP-GFP-LC3B confirmed increased autophagic flux following EPI treatment. MDA-MB-231 or SUM159PT cells stably expressing mRFP-GFP-LC3B protein were treated with either 100 nM EPI for 48 hrs or 10 nM BAF for 5 h. Red puncta (autolysosomes; arrows) indicate increased autophagic flux in response to EPI. Yellow puncta (autophagosomes; arrowheads) indicate inhibition in autophagy at the lysosomal fusion step in response to BAF. Scale bar = 10 µm. RFP positive only red puncta (# = red puncta minus green puncta) in each cell was determined and bar graphs are representative of 100 cells.

D. EPI treatment induces ER stress in MDA-MB-231 TNBC cells. MDA-MB-231 cells were treated with 100nM EPI for 6 hrs or 6 days. Levels of ER stress proteins EIF2AK3 (PERK) and HSPA5 (GRP78/BiP) were determined using immunoblot analysis. The image shown is representative of 3 biological replicates.
Figure 2. TNBC cells resistant to epirubicin have higher levels of autophagic flux relative to parent cells.

A. Epirubicin resistant R8 and SUM159PT-R75 (R75) lines have higher IC₅₀ values compared to their parental lines (p < 0.0001). EPI resistant sub-lines R8 and R75 were evaluated for response (48 hrs post-treatment) to increasing concentrations of epirubicin using the Alamar blue-based cell viability assay. Results shown are representative of at least 3 independent experiments.

B. MDA-MB-231-R8 cells are resistant to epirubicin. The R8 cell line, derived from the parental MDA-MB-231 line, was evaluated for resistance to epirubicin by the trypan blue viability assay. Error bars represent SD of 3 independent experiments.

C. Anthracycline-resistant lines R8 and R75 showed higher levels of basal autophagic flux relative to their respective parent lines. Basal level autophagy flux of MDA-MB-231 vs R8 and SUM159PT vs R75 was evaluated by LC3B-II western blot assay using saturating concentrations of BAF (5 h). Numbers below western blots indicate corresponding results of densitometry quantitation of LC3B-II/Actin. Results shown are representative of 3 independent experiments. P = Parent; R = Resistant

D. mRFP-GFP-LC3B assay for autophagic flux in TNBC lines. Cell lines stably expressing the mRFP-GFP-LC3B fusion protein were created from MDA-MB-231, SUM159PT, R8, and R75. A clear increase in autophagic flux (relatively high levels of red puncta in resistant lines R8 and R75 was observed compared to their parent lines MDA-MB-231 and SUM159PT, respectively. In parent lines, the majority of mRFP-GFP-LC3B fusion protein expression was cytoplasmic, and appeared as diffuse green-yellow staining. Scale bar = 10 µm. RFP positive only red puncta (# = red puncta minus green puncta) in each cell was determined and bar graphs are representative of 100 cells.

Figure 3. Combination treatment of autophagy inhibition and epirubicin enhances the loss in cell viability.

A. ATG-siRNA treatment of MDA-MB-231 and SUM159PT showed effective knockdown of autophagy protein targets. MDA-MB-231 and SUM159PT were transfected with siRNA alone or transfected first with siRNA for 24hrs and then treated with EPI (25 nM) for an additional 24 hrs. The siRNAs used were scramble siRNA (scr), ATG7-siRNA (two distinct siRNA sequences, ATG7-1 and ATG7-2), and ATG5-siRNA (ATG5-1). Autophagy protein knockdown was assessed by western blot analysis. Results shown are representative of 2 independent experiments.
Autophagy inhibition by ATG-siRNA sensitized TNBC cells to EPI treatment. Cells treated as indicated in figure 3A were assessed for viability by the trypan blue assay. P values shown were calculated for combined treatment of Atg5 or ATG7-siRNA + EPI compared to scr siRNA + EPI. Results shown are representative of 3 independent experiments.

Clonogenic recovery assay confirmed that combination therapy with anthracyclines and the pharmacological autophagy inhibitor CQ was most effective in reducing viability of MDA-MB-231 cells. Cells were treated with EPI or Doxorubicin (DOX), CQ or EPI/DOX+CQ (at indicated concentrations) for 4 days and grown in media without drugs for another 5 days. Colonies were visualized with crystal violet staining. Retained crystal violet staining was measured by A590 to generate a proliferation index. Error bars represent SD from 3 independent experiments.

Figure 4. Autophagy inhibition reduces viability and augments cell death of TNBC lines resistant to EPI.

Pharmacologic inhibition of autophagy sensitizes EPI-resistant TNBC cells. R8 cells resistant to EPI were treated with either CQ (6 µM) or EPI (100 nM) alone or in combination. Percent viable cells were determined 7 days after treatment. Error bars represent the SD of 3 independent experiments. Western blot below shows that the combined treatment of CQ and EPI showed the highest accumulation of LC3B-II levels in R8 cells.

Autophagy inhibition by CQ sensitizes anthracycline resistant TNBC line to EPI treatment. R8 cells were treated with EPI alone (50 or 100 nM), CQ alone (6 µM) or EPI + CQ combination for 5 days and grown in fresh media without drugs for another 5 days. Viable cells forming colonies were visualized using crystal violet staining. Retained crystal violet staining was measured at A590 to generate a proliferation index that confirmed the combination treatments in R8 cells are most effective. Error bars represent SD from three independent experiments.

ATG-siRNA treatment can effectively augment cell death in anthracycline-resistant TNBC lines. R8 and R75 cell lines were treated twice with siRNAs targeting ATG5 or ATG7. Error bars represent SD of three independent experiments. Cells were evaluated for inhibition of autophagy by immunoblot analysis (LC3B-II levels) in the presence and absence of 10nM BAF. Scramble (scr) siRNA served as controls.

Inhibition of autophagy with ATG-siRNA induces caspase-dependent cell death. EPI resistant TNBC cells were assayed for induction of caspase 3/7 activity using the luminenscence (RLU = Relative luminescence unit; y axis)-based Caspase-Glo assay.
Error bars represent SD from 6 replicates derived from 2 independent experiments. Increased caspase activity following ATG-siRNA treatments was confirmed by determining cleaved PARP levels (89kDA) using immunoblot assays. Images shown are representative of 3 biological replicates. Cychloheximide (CHX) and staurosporine (Stauro) were used as control caspase activators.

Figure 5. Assessment of *in vivo* autophagy-related protein levels in MDA-MB-231 xenograft tumors.

A. Tumors treated with HCQ show increased accumulation of LC3B-II compared to controls. Protein lysates from individual MDA-MB-231 xenograft tumors that were harvested 4 h following treatment with saline (n=4) or bolus I.P.HCQ 120mg/kg (n=4) were analyzed by western blot for LC3B, and tubulin protein expression. Corresponding mean LC3B-II/tubulin values (per treatment group) were determined using densitometry analysis ; error bars indicate SD.

B. MDA-MB-231 xenograft tumors treated with HCQ, EPI, or their combination show higher accumulation of LC3B compared to Saline controls. IHC analysis for LC3B protein expression was performed on representative samples from each of the following treatment groups: Saline, HCQ (I.P. 90mg/kg daily, 5days/week), EPI (I.V. 7mg/kg, weekly), and HCQ (90mg/kg)+EPI (7mg/kg). The treatment was given for 3 wk. For each sample, histological (H&E) section is also presented (original magnification x10 for all sections).

Figure 6. Combinatorial EPI+HCQ treatment reduces growth in MDA-MB-231 and MDA-MB-231-R8 tumor xenograft models.

A. Tumor growth curves of MDA-MB-231 xenografts following initiation of treatment with Saline (n=24), Epi7mg/kg (n=12), HCQ90mg/kg (n=6), or Epi7mg/kg + HCQ90mg/kg combination (n=6). The relative tumor volumes, combined from 4 independent *in vivo* studies, were normalized to their original sizes, so that the initial tumor volume in each animal (Day 0, before the treatment start) was set as 1, and relative tumor volume, evaluated twice a week for 3 weeks, was plotted as the fold change relative to day 0. At each time point, the data represent mean values and error bars indicate SEM of n tumors in each group. The combination of Epi7mg/kg with HCQ90mg/kg resulted in a significant tumor growth inhibition compared to saline controls and Epi7mg/kg alone. The number of asterisks above the respective data points indicate statistical significance between the combination group and either EPI-treated group or saline control (*p<0.05,**p<0.01, ***p<0.0001; Kruskal-Wallis test with Dunn’s correction for multiple comparisons).
Kaplan-Meier plots for each treatment group. Percentage survival at each time point is defined as the percentage of animals with less than 50% increase in tumor size; n = 6 (HCQ90 mg/kg and HCQ90mg/kg+Epi7mg/kg), 12 (Epi7mg/kg), and 24 (Saline) animals at day 0. Median growth time (MGT) was 7, 15, and 18 days for saline controls, Epi7mg/kg alone, and HCQ90mg/kg alone, respectively; none of the HCQ90mg/kg+Epi7mg/kg-treated tumors reached 1.5x original size during the treatment period. Log-rank (Mantel-Cox) test indicated that HCQ90mg/kg+Epi7mg/kg treatment resulted in a significant increase in MGT compared to saline controls (p < 0.0001) and Epi7mg/kg alone (p < 0.05). HCQ90mg/kg treatment alone significantly increased MGT compared to saline controls (p < 0.005) and had similar effect (p=0.95) to Epi7mg/kg alone.

B Tumor growth curves of MDA-MB-231-R8 xenografts following initiation of 4 week treatment with Saline (n=15), Epi7mg/kg (n=15), HCQ90mg/kg (n=15), or Epi7mg/kg+HCQ90mg/kg combination (n=14). The relative tumor volumes, combined from 2 independent in vivo studies, were normalized to their original sizes (Day 0, before the treatment start). At each time point, the data represent mean values (fold change in tumor size) and error bars indicate SEM of n tumors in each group. The combination of Epi7mg/kg with HCQ90mg/kg resulted in a significant tumor growth inhibition compared to saline controls (p<0.05, Kruskal-Wallis test with Dunn’s correction for multiple comparisons).

Kaplan-Meier plots for each treatment group. Percentage survival at each time point is defined as the percentage of animals with less than 50% increase in tumor size; n = 15 (Saline, HCQ90 mg/kg and Epi7mg/kg groups) and 14 (Epi7mg/kg+HCQ90mg/kg group) animals at day 0. Median growth time (MGT) was 13 days for saline controls and Epi7mg/kg alone group, 16 days for HCQ90mg/kg alone group, and 24 days for the HCQ90mg/kg+Epi7mg/kg-treated tumors; half of the mice in the combination therapy group had less than 50% increase in tumor volume by the end of the study. Log-rank (Mantel-Cox) test indicated that HCQ90mg/kg+Epi7mg/kg treatment resulted in a significant increase in MGT compared to saline controls (p = 0.0002) and Epi7mg/kg alone (p =0.0042).

C. R8 xenograft tumors show higher accumulation of LC3B under basal conditions than MDA-MB-231 tumors. IHC for LC3B (at x10 and x63 magnifications) and histological (H&E) sections are shown for the representative tumors from each group.
Figure 1

A. Representative images of cell morphology showing the effects of different concentrations of epirubicin (EPI) on cell viability. 

B. Western blot analysis of LC3 and Actin expression in MDA-MB-231 and SUM159PT cells treated with EPI and BAF. 

C. Confocal microscopy images of MDA-MB-231 and SUM159PT cells treated with EPI and BAF, showing changes in red punctae per cell. 

D. Western blot analysis of EIF2AK3 and HSPA5 expression in MDA-MB-231 cells treated with EPI and BAF.
Figure 3
Figure 4
Figure 5

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**B**

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Fold Change in Expression

- **Saline**
- **HCQ**

*P < 0.01*
Figure 6

A

B

C

MDA-MB-231    R8

LC3B

H&E

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Autophagy Inhibition Augments the Anticancer effects of Epirubicin treatment in Anthracycline-Sensitive and Resistant Triple Negative Breast Cancer

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