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

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Abstract

Purpose: Triple-negative breast cancers (TNBC) are defined by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/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 used in vitro and in vivo models of TNBC using cell lines sensitive to epirubicin 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 epirubicin induced autophagic flux in TNBC cells. Epirubicin-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 epirubicin with the autophagy inhibitor hydroxychloroquine resulted in a significant reduction in tumor growth compared with monotherapy with epirubicin.

Conclusion: Autophagy inhibition enhances therapeutic response in both anthracycline-sensitive and -resistant TNBC and may be an effective new treatment strategy for this disease. Clin Cancer Res; 20(12); 3159–73. ©2014 AACR.

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% to 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 histopathologic features (2). Because of 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, patients with TNBC have poorer outcomes due to drug resistance and more aggressive recurrent disease compared with 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. Because of 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-
Translational Relevance

Triple-negative breast cancers (TNBC) are defined by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (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 induced autophagy as a prosurvival 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 epirubicin treatment, both in sensitive and resistant TNBC cells in vitro. In TNBC tumor xenograft mouse models, combined treatment of epirubicin and hydroxychloroquine significantly reduced the growth of both anthracycline-sensitive and -resistant TNBC tumors, compared with either monotherapy. These data thus provide a rationale for clinical evaluation of hydroxychloroquine in combination with anthracycline therapy in advanced TNBC.

Materials and Methods

Reagents

The following antibodies were used: anti-LC3B (Abcam), anti-β-actin (Abcam), anti-ATG7 (Novus), anti-ATG5 (Cell Signaling Technology), anti-p62 (BD Biosciences), anti-PERK (E3F2AK3; Cell Signaling Technology), anti-BiP (HSPA5; Cell Signaling Technology), anti-PARP (Cell Signaling Technology), anti-BiP, and goat anti-rabbit IgG–HRP (Santa Cruz Biotechnology). The following reagents were used: hydroxychloroquine (Acros OrganicsSource), chloroquine (Sigma-Aldrich), bafilomycin A1 (Sigma-Aldrich), epirubicin (EPI; Pharmaceutical Partners of Canada Inc.), doxorubicin (Pfizer Canada Inc.), and mitoxantrone (Hospira Healthcare Corporation).

Cell lines and reagents

For in vitro experiments, MDA-MB-231 cells [2005; ATCC authentication by isoenzyme and short tandem repeat (STR) analyses] were maintained at 37°C, 5% CO₂ in Gibco DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS). For 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°C, 5% CO₂ in Ham’s F-12 medium with 10% FBS, HEPES (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), and 1 μg/mL hydrocortisone (Sigma-Aldrich). BT549 cells [2011; ATCC authentication by isoenzyme and STR analyses] were maintained at 37°C, 5% CO₂ in media with supplements as indicated in the Materials and Methods section.
Gibco DMEM (Life Technologies) supplemented with 10% FBS and 5 μg/mL insulin (Sigma-Aldrich).

Resistant cell lines MDA-MB-231-R8 and SUM159PT-R75 were generated by growing in increasing concentrations (12.5–100 nmol/L for R8; 100 nmol/L–1 μmol/L for R75) of epirubicin for approximately 1 year and 6 months, respectively, and were maintained in culture with 100 nmol/L and 1 μmol/L epirubicin, respectively. Before any experiments, the cells were withdrawn from epirubicin for at least two 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 (mean ± SEM) and calculated using nonlinear 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 × 10⁴ cells were plated for 24 hours in Lab-Tek II Chamber Slides (Nalge Nunc). MDA-MB-231 and SUM159PT cells were treated with 100 and 200 nmol/L epirubicin, respectively, for 48 hours. To inhibit flux, 15 and 25 nmol/L bafilomycin A1 (for MDA-MB-231 and SUM159PT, respectively) was added 5 hours 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 with the Caspase-Glo 3/7 Kit (Promega) and transfected with scramble or ATG-siRNAs. Staurosporine or cycloheximide served as controls. Ninety-six hours after the first transfections, caspase levels were determined with the Caspase-Glo 3/7 Kit. Luminescence was measured with a Synergy H4 Hybrid (BioTek).

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

Cells were plated at 1 × 10³ cells per well in a 6-well plate. After 24 hours, drugs were added and cells were incubated for 4 days. Media with drug were replaced with fresh media without drug and cultured for another 6 to 7 days. Colonies were fixed with 4% PFA and stained with 0.1% 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 minutes. Readings were taken at A₅₉₀ 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 epirubicin, cells were plated at 2 × 10⁵ per 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 25 nmol/L epirubicin. Twenty-four hours after treatment, cell viability and Western blot analyses were performed.

For treatments with siRNA only, cells were transfected under serum-free conditions followed by the addition of serum (10% FBS, final) after 16 hours. Forty-eight hours after the initial siRNA transfection, cells were transfected again with respective siRNAs under serum-free conditions. Media with serum were added after the second transfection and cells were harvested 112 hours from the time of the first siRNA transfection.

**Western blot analysis**

Protein lysates were prepared using the RIPA Lysis Buffer Kit (Santa Cruz Biotechnology), 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. Quantification of the signal was performed using ImageQuant 5.1 software or Bio-Rad image analysis software. Autophagic flux assay was performed with lysosomal inhibitor bafilomycin and Western blot analysis (details in Supplementary Methods; ref. 27).

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

MDA-MB231-R8 or SUM159PT-R75 cells (8 × 10⁴) 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-six hours after the first transfections, caspase levels were determined with the 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 (Vancouver, BC, Canada) before 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 = 6–10 per treatment group) were used to assess tumor growth and autophagy markers. MDA-MB-231 or MDA-MB-231-R8 cells (1 × 10⁶ in Matrigel) were injected subcutaneously in 9- to 14-week-old mice, and tumor growth was monitored. Once the average tumor volume reached approximately 100 mm³, mice were randomized into treatment groups. To evaluate the immediate effects of hydroxychloroquine on autophagy levels in tumors, a bolus of hydroxychloroquine at a relatively high dose, 120 mg/kg, was administered by intraperitoneal injection in tumor-bearing mice. At 2, 4, and 24 hours after...
injection, the tumors were harvested and analyzed for LC3B-II and p62 levels. To determine the therapeutic efficacy of epirubicin, hydroxychloroquine, or their combination, mice were treated for up to 4 weeks with saline, maximum-tolerated dose (MTD) of epirubicin (i.v. 7 mg/kg, weekly), hydroxychloroquine (i.p. 90 mg/kg, daily × 5 d/wk), or both. Tumor size measurements with calipers as well as monitoring for signs of toxicity (e.g., body weight loss and behavioral changes) were performed twice a week and daily, respectively. Four hours after the last injection of hydroxychloroquine, animals were terminated, and a necropsy was performed to evaluate drug toxicity. Each harvested tumor was used for Western blot analysis of LC3B-II and p62 levels and immunohistochemical (IHC) analysis (see below).

**Hematoxylin and eosin 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 for IHC or Hematoxylin and eosin (H&E) staining. For IHC, the LC3B antibody from Abcam (cat# ab48394) was stained on the Ventana DiscoveryXT (details in Supplementary Data).

**Statistical analyses**

For in vitro cell viability or proliferation assays comparing combination treatment to epirubicin treatment alone, Student t test (two-tailed, unpaired) was used. For multiple comparisons (casapse assay), one-way ANOVA with Dunnett post-test was used.

Statistical significance of differences in tumor measurements between groups was calculated using the Kruskal–Wallis test with Dunn correction for multiple comparisons. For survival analysis, a log-rank (Mantel–Cox) test was used. 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 epirubicin on TNBC cells, MDA-MB-231 and SUM159PT cells were treated with epirubicin at varying concentrations. Epirubicin treatment (≥100 nmol/L) affected the morphology of MDA-MB-231 cells after 48 hours; cells appeared increased in size with enlarged nuclei (Fig. 1A). At 400 nmol/L epirubicin, a reduction in cell numbers was clearly observed under the inverted microscope (Fig. 1A). Cell counts by the Trypan blue cell exclusion assay following 48 hours of 100 and 400 nmol/L epirubicin treatment showed 46% ± 9.6% and 17% ± 2.9% viable cells, respectively, compared with the no treatment control (Fig. 1A).

To determine whether epirubicin treatment increases autophagic flux in TNBC cells, we conducted analyses using autophagy protein MAP1LC3B (LC3B) and the lysosomal inhibitor bafilomycin A1 (details in Materials and Methods) at its established saturating concentrations (Supplementary Fig. S1). As indicated by increased levels of LC3B-II in the presence of bafilomycin A1, and as shown in Fig. 1B in MDA-MB-231 and SUM159PT cells, increased autophagic flux was observed from 6 hours and up to 6 days after drug treatments. These results show that epirubicin treatment induces autophagic flux, and that high levels of autophagy are maintained in the remaining cells that survive epirubicin treatment. Consistent with this finding, we observed that epirubicin treatment also leads to increased transcript levels of autophagy genes in TNBC cells that survived epirubicin treatment (Supplementary Fig. S2A and S2B).

To confirm our results, we used an alternate flux assay using an mRFP-GFP-LC3B construct stably integrated in MDA-MB-231 and SUM159PT cells (see ref. 28 and Materials and Methods). As shown in Fig. 1C, both MDA-MB-231 and SUM159PT cells treated with epirubicin showed increased RFP-positive red puncta compared with untreated cells (P < 0.001), indicating that epirubicin treatment induced autophagic flux. Control cells treated with bafilomycin A1 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 analysis, these results show that epirubicin induces autophagic flux in TNBC cells. To identify potential molecular mechanisms related to epirubicin treatment effects, we determined the levels of endoplasmic reticulum stress proteins HSPA5 (GRP78/Bip) and EIF2AK3 (PERK) at 6 hours and 6 days after epirubicin treatment in MDA-MB-231 cells (Fig. 1D). Both proteins showed elevated levels following epirubicin 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 epirubicin. Compared with the parental MDA-MB-231 epirubicin-sensitive line, the derivative MDA-MB-231-R8—resistant line (hereafter referred to as R8) had an 8-fold higher IC50 [MDA-MB-231 IC50 (95% confidence interval (CI)), 515 nmol/L (389–683 nmol/L) versus R8 IC50 (95% CI), 4,272 nmol/L (3,155–5,783 nmol/L); 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 with the sensitive parental line [SUM159PT IC50 (95% CI), 56 nmol/L (35–90 nmol/L) versus R75 IC50 (95% CI), 8,251 nmol/L (5,954–11,434 nmol/L; P ≤ 0.0001; Fig. 2A)]. The parental line SUM159PT seems to be more sensitive to epirubicin compared with MDA-MB-231; however, the derived R75-resistant line was more resistant to epirubicin compared with 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 100 and 200 nmol/L epirubicin treatment, respectively, compared with their parental cells, which showed
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 bafilomycin A1 (Supplementary Fig. S1; 10 and 40 nmol/L 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 etoposide-resistant lines showed higher levels of red puncta compared with their respective parental lines (Fig. 2D).
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 IC_{50} values compared with their parental lines (P < 0.0001). Epirubicin-resistant sublines R8 and R75 were evaluated for response (48 hours after treatment) to increasing concentrations of epirubicin using the Alamar blue-based cell viability assay. Results shown are representative of at least three 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, SD of three 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 versus R8 and SUM159PT versus R75 was evaluated by LC3B-II Western blot analysis using saturating concentrations of bafilomycin A1 (5 hours). Numbers below Western blot analyses indicate corresponding results of densitometry quantitation of LC3B-II/actin. Results shown are representative of three 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 with 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. *, P < 0.0001; Student t test.
indicating an increase in autophagic flux. These experiments show that the anthracycline-resistant lines R8 and R75 have elevated levels of basal autophagy compared with their respective parental lines.

Chemotherapy in combination with autophagy inhibition is more effective than monotherapy in reducing viability of both epirubicin-sensitive and -resistant TNBC cells in vitro

To determine whether autophagy inhibition sensitizes cells to epirubicin, we specifically inhibited autophagy with siRNAs targeting essential autophagy-related (ATG) proteins ATG7 or ATG5 in combination with a low concentration of epirubicin. Knockdown of ATG targets was confirmed using Western blot analysis (Fig. 3A). The effects of ATG knockdown with and without epirubicin on cell viability were assessed by Trypan blue assays. ATG7-siRNA or ATG5-siRNA combined with epirubicin 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 with MDA-MB-231 cells treated with epirubicin and control scramble siRNA (Fig. 3B). As shown in Fig. 3B, combined treatment of autophagy inhibition with ATG7-siRNA or ATG5-siRNA and epirubicin 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 with controls. These results suggest that in TNBC cells, the knockdown of ATG7 or ATG5 further decreases cell viability upon epirubicin treatment.

Because pharmacologic inhibition of autophagy with chloroquine or hydroxychloroquine is currently being tested in clinical trials, we evaluated the combination effects of chloroquine and epirubicin on cell recovery and proliferation using MDA-MB-231 cells. We performed clonogenic crystal violet recovery assays where cells were treated with sublethal concentrations of epirubicin (12.5 nmol/L) and chloroquine (3 or 6 μmol/L), either alone or in combination. The clonogenic crystal violet assay showed 36% ± 25% proliferating cells for chloroquine (6 μmol/L) and 60% ± 17% proliferating cells for epirubicin treatments compared with untreated cells. Combination of 6 μmol/L chloroquine and 12.5 nmol/L epirubicin was most effective in reducing proliferating cells with 9.6% ± 8% remaining cells compared with no treatment (Fig. 3C). The effect of epirubicin and chloroquine combination treatment was also determined in the TNBC cell line BT549, which showed a 43% ± 11% reduction in viability compared with epirubicin treatment alone (Supplementary Fig. S3). To determine whether other anthracyclines in combination with chloroquine may effectively reduce clonogenicity of TNBC cells compared with anthracycline treatment alone, we treated MDA-MB-231 cells with doxorubicin alone, chloroquine alone, or in combination and performed crystal violet recovery assays. Our data showed that combination of doxorubicin + chloroquine was most effective in reducing clonogenicity with 33% ± 6% proliferating cells compared with 69% ± 6% and 66% ± 23% proliferating cells for doxorubicin or chloroquine alone, respectively (Fig. 3C). These results indicate that anthracyclines or chloroquine treatments alone can significantly reduce clonogenicity of MDA-MB-231 cells but the combination of anthracyclines and chloroquine can more effectively eliminate cells. These results taken together indicate that either genetic or pharmacologic 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 epirubicin and chloroquine is also more effective compared with epirubicin alone in epirubicin-resistant cells, the resistant cell lines were treated with sublethal 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 chloroquine and epirubicin (Fig. 4A) compared with epirubicin treatment alone. Similar results were obtained with R75 cells, where the combined treatment showed 22% ± 11% (P < 0.025) reduction in viability compared with epirubicin alone (Supplementary Fig. S3B). Cells treated with chloroquine+epirubicin showed high levels of accumulated LC3B-II protein, indicating that 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 chloroquine, epirubicin, or chloroquine+epirubicin and performed the crystal violet recovery assay. As expected, the resistant R8 cells treated with chloroquine alone recovered and produced large clones. The largest reduction in clonogenicity was observed with chloroquine+epirubicin treatment (Fig. 4B). The proliferation recovery assay (Fig. 4B) showed significant reduction in proliferating cells for chloroquine+epirubicin treatment compared with epirubicin treatment alone (P < 0.02).

To determine whether specific autophagy inhibition reduces the viability of epirubicin-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 with 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 bafilomycin A1 (Supplementary Fig. S1). As shown for R8 cells (Fig. 4C), compared with scramble controls, cells treated with ATG5-siRNA or ATG7-siRNA led to reduced LC3B-II levels with or without bafilomycin A1, 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 caspase-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 with 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...
Figure 4. Autophagy inhibition reduces viability and augments cell death of TNBC lines resistant to epirubicin. A, pharmacologic inhibition of autophagy sensitizes epirubicin-resistant TNBC cells. R8 cells resistant to epirubicin were treated with either chloroquine (6 μmol/L) or epirubicin (100 nmol/L) alone or in combination. Percentages of viable cells were determined 7 days after treatment. Error bars, SD of three independent experiments. Western blot analysis below shows that the combined treatment of chloroquine and epirubicin showed the highest accumulation of LC3B-II levels in R8 cells. B, autophagy inhibition by chloroquine sensitizes anthracycline-resistant TNBC line to epirubicin treatment. R8 cells were treated with epirubicin (EPI) alone (50 or 100 nmol/L), chloroquine (CQ) alone (6 mmol/L), 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 that the combination treatments in R8 cells are most effective. Error bars, SD from three independent experiments. C, 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, SD of three independent experiments. Cells were evaluated for inhibition of autophagy by immunoblot analysis (LC3B-II levels) in the presence and absence of 10 nmol/L bafilomycin A1. Scramble (scr) siRNA served as controls. D, inhibition of autophagy with ATG-siRNA induces caspase-dependent cell death. Epirubicin-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, SD from six replicates derived from two independent experiments. Increased caspase activity following ATG-siRNA treatments was confirmed by determining cleaved PARP levels (89 kDa) using immunoblot assays. Images shown are representative of three biologic replicates. Cycloheximide (CHX) and staurosporine (Stauro) were used as control caspase activators.
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 inhibition of autophagy contributes to caspase-dependent cell death in epirubicin-resistant TNBC cells.

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

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

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

The in vivo efficacy of epirubicin and hydroxychloroquine alone and in combination was assessed at their MTDs (7 and 90 mg/kg, respectively). MDA-MB-231 tumor growth in mice treated with epirubicin or hydroxychloroquine was moderately reduced compared with saline ($P = 0.0025$ and 0.017, respectively); however, the tumors continued to increase in size. In contrast, tumor growth in mice treated with the combination of epirubicin and hydroxychloroquine was significantly reduced compared with saline (by more than 50%; $P < 0.0001$) or monotherapy with epirubicin (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 the 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, epirubicin alone, and hydroxychloroquine alone, respectively; none of the hydroxychloroquine + epirubicin–treated tumors reached 1.5× original size during the treatment period. Log–rank (Mantel–Cox) test indicated that the combination treatment resulted in a significant increase in MGT compared with saline controls ($P < 0.0001$) and epirubicin alone ($P < 0.05$). Hydroxychloroquine treatment alone significantly increased MGT compared with saline controls ($P < 0.005$) and had a similar effect ($P = 0.95$) to epirubicin alone.

The toxicity of the treatment regimens described above was mild. Less than 10% 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 hydroxychloroquine–epirubicin combination can be given safely at a dose that augments antitumor activity of epirubicin in epirubicin-sensitive TNBC.

To follow-up on our in vitro results suggested that anthracycline-resistant cancer cells could be sensitized to chemotherapy by autophagy inhibition, we treated mice bearing MDA-MB-231-R8 tumors with epirubicin, hydroxychloroquine, or their combination. Therapy with either hydroxychloroquine or epirubicin had minimal or no effect on MGT when used as single agents (Fig. 6B). However, combinatorial treatment with epirubicin and hydroxychloroquine significantly reduced tumor growth compared with saline controls ($P < 0.05$). MGT was 13 days for the saline and epirubicin alone groups, 16 days for the hydroxychloroquine alone group, and 24 days for the epirubicin + hydroxychloroquine group ($P < 0.01$, log-rank test). Half of the mice (7 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% body weight loss was noted in all treatment groups. These results indicate that hydroxychloroquine sensitizes anthracycline-resistant tumors to epirubicin, and the combination therapy with hydroxychloroquine and epirubicin 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 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 unknown. Epirubicin is a structural analog of doxorubicin that is commonly used to treat TNBC. Epirubicin, however, is usually better tolerated (29). To our knowledge, this is the first report to demonstrate the effectiveness of autophagy inhibition in combination with epirubicin in both anthracycline-sensitive and -resistant TNBC cells in vitro and in vivo.

It is demonstrated that treatment of epirubicin-sensitive TNBC cells with epirubicin (100–200 nmol/L) induces autophagic flux that functions to promote cell survival. A previous study showed that high-dose epirubicin treatment (2.5 μmol/L) 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 in vivo and observed a significant reduction in xenograft tumor growth when we combined hydroxychloroquine (90 mg/kg) with 7.0 mg/kg (weekly) of epirubicin (see Fig. 6). In humans, several phase II studies have used epirubicin as a single agent at doses between 12 and 30 mg/m$^2$ (weekly) to treat advanced breast cancer (31) and higher doses of 90 mg/m$^2$ have also been reported (32). Using the FDA guidelines for converting animal doses to human doses, the 7.0 mg/kg epirubicin in mice converts to 20 mg/m$^2$ in humans and therefore represents a clinically relevant range (33). In another study involving patients with locally advanced primary breast cancer, four cycles of epirubicin at 90 mg/m$^2$ given every 3 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 1,264, 13.3, and 11.2 ng/mL, respectively (32). Circulating plasma levels declined rapidly to less than 100 ng/mL by 2 hours after epirubicin administration. The concentrations of epirubicin used in our in vitro studies were typically in the range of 12.5 to 200 nmol/L, equivalent to 7.3 to 116 ng/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 epirubicin-resistant sublines, derived from sensitive TNBC lines, had relatively high levels of basal autophagic flux (Fig. 2C and D). 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 that 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 with their sensitive parental lines has been reported previously in breast cancer cell lines (12, 17, 34) and other cancer (35) cell lines. Our IHC...
Figure 6. Combinatorial epirubicin (EPI) and hydroxychloroquine (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 \( (n = 24) \), epirubicin 7 mg/kg \( (n = 12) \), hydroxychloroquine 90 mg/kg \( (n = 6) \), or epirubicin 7 mg/kg + hydroxychloroquine 90 mg/kg combination \( (n = 6) \). The relative tumor volumes, combined from four 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 epirubicin 7 mg/kg with hydroxychloroquine 90 mg/kg resulted in a significant tumor growth inhibition compared with saline controls and EPI 7 mg/kg alone. The number of asterisks above the respective data points indicate statistical significance between the combination group and either epirubicin-treated group or saline control \( (* P < 0.05; ** P < 0.01; *** P < 0.0001; \text{Kruskal–Wallis test with Dunn correction for multiple comparisons}) \). 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; \( n = 6 \) (hydroxychloroquine 90 mg/kg and hydroxychloroquine 90 mg/kg + epirubicin 7 mg/kg), 12 (epirubicin 7 mg/kg), and 24 (Saline) animals at day 0. MGT was 7, 15, and 18 days for saline controls, epirubicin 7 mg/kg alone, and hydroxychloroquine 90 mg/kg alone, respectively; none of the hydroxychloroquine 90 mg/kg + epirubicin 7 mg/kg–treated tumors reached 1.5\( \times \)original size during the treatment period. Log-rank (Mantel–Cox) test indicated that hydroxychloroquine 90 mg/kg + epirubicin 7 mg/kg treatment resulted in a significant increase in MGT compared with saline controls \( (P < 0.0001) \) and epirubicin 7 mg/kg alone \( (P < 0.05) \). Hydroxychloroquine 90 mg/kg treatment alone significantly increased MGT compared with saline controls \( (P < 0.005) \) and had similar effect \( (P = 0.95) \) to epirubicin 7 mg/kg alone. (Continued on the following page.)
studies of parental and R8 line xenograft tumors (see Fig. 6C) showed elevated levels of LC3B protein in R8 tumors compared with the MDA-MB-231 parental tumors, suggesting that resistant tumors also maintain higher levels of autophagy in vivo even when the selective pressure (epirubicin) 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 with other molecular subtypes of breast cancer (36) and high LC3B was associated with progression and poor outcome in TNBC (37). 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 pharmacologic aggravators of endoplasmic reticulum stress in TNBC (19, 38) and in combination with various drugs in other types of breast cancer cells (12, 17, 30, 39) is known to enhance cell death. These findings are consistent with our results where caspase activation and increased PARP cleavage were clearly observed in epirubicin-resistant R8 and R75 cells following knockdown of autophagy with ATG-siRNAs (see Fig. 4). In the sensitive cells, we found that epirubicin treatment led to increased levels of endoplasmic reticulum stress proteins (Fig. 1D). Because endoplasmic reticulum stress is a known inducer of autophagy and cell death (40), it is possible that the observed effects of epirubicin 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, baflomycin A1, chloroquine, or hydroxychloroquine. 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 TP53 and KRAS or HRAS, and evidence indicates that alterations in these proteins may affect dependency on the autophagy process for survival and influence sensitivity to hydroxychloroquine (41–44). Hydroxychloroquine may also exert additional effects, such as immunomodulation and increased drug cytotoxicity, due to changes in lysosomal pH (45, 46). Although hydroxychloroquine (i.e., a chloroquine derivative) may alter processes other than autophagy, our in vitro studies using either chloroquine or ATG-siRNAs clearly show that autophagic function is impaired following treatment of cells with these agents (see Fig. 4). Chloroquine had similar effects to ATG5 or ATG7-siRNA in cells in vitro, suggesting that autophagy inhibition is at least in part responsible for the reduced viability observed. Additional studies investigating genetic backgrounds, along with the ongoing clinical trials, will help determine the underlying factors and the extent of differential responses to hydroxychloroquine in patients with cancer.

Although our results showed that combination therapy, relative to monotherapy of either hydroxychloroquine or epirubicin, 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 epirubicin activity in R8 tumors. We did not detect a difference in growth rate between the parental and R8 cells in vivo, 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 with parental cells. In our studies hydroxychloroquine was already administered at its MTD. It is possible that more potent lysosomal inhibitors, such as Lys05 (47), or small-molecule inhibitors targeting essential autophagy proteins could be tested in the future. Furthermore, there may be a role for drug carrier technology here, where nanoscale delivery systems could be used to ensure that drugs such as hydroxychloroquine 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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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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