Cancer Science | Japanese Cancer Association JCA | Open Access

Inhibiting autophagy increases epirubicin's cytotoxicity in breast cancer cells

Wei Guo,[1,2,3] Yu Wang,[1,3] Zhu Wang,[1] Yan-Ping Wang[1,2] and Hong Zheng[1,2]

[1]Laboratory of Molecular Diagnosis of Cancer, West China Hospital, West China Medical School, Sichuan University, Chengdu; [2]State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu, China

Key words

Autophagy, bafilomycin A1, breast cancer, cytotoxicity, epirubicin

Correspondence

Hong Zheng, Laboratory of Molecular Diagnosis of Cancer, State Key Laboratory of Biotherapy, West China Medical School, West China Hospital, Sichuan University, Chengdu, Sichuan, 610041 China.
Tel: +86-189-8060-1830; Fax: +86-028-8542-3278;
E-mail: hzheng@scu.edu.cn

[3]These authors contributed equally to this work.

Funding Information
Science and Technology Program of Sichuan Province.

Received February 12, 2016; Revised August 14, 2016; Accepted August 19, 2016

Cancer Sci **107** (2016) 1610–1621

doi: 10.1111/cas.13059

Chemotherapy, radiotherapy, and endocrinotherapy are documented to induce autophagy among breast cancer cells, but the role of autophagy in this disease has been attributed as cytoprotective as well as tumor-suppressing. Thus we studied MDA-MB-231 and SK-BR-3 breast cancer cell lines treated with epirubicin (EPI) to assess autophagy and apoptosis. We found out that EPI induced apoptosis and autophagy in both cell lines. The lysosomal inhibitor bafilomycin A1 inhibited cellular autophagy and enhanced EPI-triggered apoptosis, perhaps due to inhibition of autolysosome formation, which then inhibited autophagic effects of engulfing and clearing damaged mitochondria. This inhibition increased mitochondrial cytochrome C release which augmented epirubicin-induced caspase-dependent apoptosis and cytotoxicity. In addition, the lysosomal neutralizing agent ammonia chloride (AC), and Atg7 knockdown by siRNA, could inhibit epirubicin-triggered autophagy, enhance cytotoxicity, and increase caspase-9- and caspase-3-dependent apoptosis. Thus, autophagy plays a prosurvival role in EPI-treated MDA-MB-231 and SK-BR-3 cells, and autophagy inhibition can potentially reverse this effect and increase the cytotoxicity of EPI.

Breast cancer is the most common malignancy among women and chemotherapy is essential for care and cure.[1] Epirubicin is a frequently used drug to treat breast cancer, but resistance to EPI is common.[2–4]

Autophagy is an evolutionarily conserved lysosome-dependent degradation in eukaryotes characterized by the hydrolysis of engulfed autophagosomal materials by lysosomal acidic enzymes.[5] Autophagy is a basal activity and increases when cells are exposed to anoxia, poor nutrition, chemicals, and radiation.[6,7] Studies by others have suggested that lysosomal inhibitors, such as BAF and AC (NH_4Cl) can be used to inhibit autophagy[8–10] through the blockade of autolysosome formation and autophagosomal content degradation.[11,12] In addition, *Atg* gene knockdown could be an approach to produce autophagy inhibition.

How autophagy affects breast cancer is controversial[13]; some studies suggest that autophagy promotes type II programmed cell death,[14–17] but other reports indicate that autophagy induced in breast tumor cells is cytoprotective and reduces cell death.[18–22] Our previous work confirmed that EPI induces cytoprotective autophagy with little apoptotic death in MCF-7 cells,[21] but some reports suggest these events are cell-line specific.[23,24] MCF-7 cells have defects in caspase-3,[25,26] which is critical for apoptosis. Therefore, the study of autophagy in other breast cancer cell lines is necessary to investigate the effects of EPI.

Using MDA-MB-231 and SK-BR-3 breast cancer cell lines, which have caspase-3 expression and distinct features with respect to hormone receptors, we assessed EPI for induction of autophagy and apoptosis. Bafilomycin A1 was investigated in the cell lines for inhibition of autolysosome synthesis and blockade of autophagy triggered by EPI. Such an autophagy-inhibiting effect should increase apoptosis by promoting release of Cyt C from mitochondria and enhance cytotoxicity. Ammonium chloride and downregulation of ATG7 by siRNA were also used and similar effects to BAF.

Materials and Methods

Cell culture, reagents, and antibodies. MDA-MB-231 and SK-BR-3 breast cancer cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured at 37°C in RPMI-1640 and DMEM (Sigma-Aldrich, St Louis, USA) respectively, and supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Epirubicin (diluted in DMEM) was from Pfizer Pharmaceuticals (H20000496, New York, USA). Bafilomycin A1 (diluted in 0.5% DMSO, B1793), fluorescent dye MDC (30432), DAPI (D8417), MTT (M2128), propidium iodine (p4170), and Trypan blue (T6146) were also from Sigma-Aldrich. Kits to measure caspase-9 activity (C1157), caspase-3 activity (C1116), kits to isolate mitochondria (C3601) caspase-3 inhibitor Ac-DEVD-CHO (C1206), and One-step TUNEL Apoptosis Assay kit (C1088) were from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-LC3 polyclonal antibody (PM036) was from MBL Co. Ltd (Nagoya, Japan). Mouse anti-GAPDH mAb (200306-7E4), mouse anti-COX IV mAb (200147), rabbit anti-Atg7 pAb (500691), and rabbit anti-p62 pAb (614662) were from Zen Bioscience (Chengdu, China). Mouse anti-β-actin (C4) mAb was from Santa Cruz Biotechnology (Sc-47778, Dallas, USA). The si-Atg7 (with sequence 5′-GGUCAAAGGACGAAGAUAATT UUAUCUUCGUCCUUU GACCTT-3′) and control siRNA were synthesized by GenePharma (Shanghai, China) and the primer (forward primer, agttgtttgcttccgtgac; reverse primer, tgcctcctttctggttctt) for detecting si-Atg7's interference efficiencies through RT-qPCR was synthesized by Sangon Biotech (Shanghai, China).

Cytotoxicity assay through MTT. MDA-MB-231 and SK-BR-3 cells were seeded in 96-well flat-bottomed plates at 8×10^3 and 1.2×10^4 per well, respectively. Medium containing various concentrations of BAF were added and cultured at 37°C for 24, 48, and 72 h. Inhibition was measured to select for subsequent experiments the appropriate dose of BAF and exposure time that inhibited autophagy without cytotoxicity.

Monodansylcadaverine sequestration assay. Cells were seeded at 5×10^5 (MDA-MB-231) and 7×10^5 (SK-BR-3) in 6-well flat-bottomed plates, and cultured for 24 h at 37°C. The cells were then treated with EPI, BAF, EPI + BAF (EPI concentration was IC_{50} at 48 h for each cell line), and plain medium added to the control group (control group in all other assays were also treated with plain medium). All the cells were then cultured for 48 h in total at 37°C. During the incubation an additional dose of BAF was added to the BAF and EPI + BAF groups 24 h before harvesting cells. All cells were treated with 0.1 mM MDC for 1 h at 37°C counted, and then collected at same amount via centrifugation at 600 g for 5 min. Then 400 μL lysis buffer was added to samples that were incubated on ice with light agitation for 30 min and centrifuged at 13,000 g for 10 min to extract the supernatant. Fluorescence of the supernatant was measured at 535 nm (emission) and 340 nm (excitation) with a microplate fluorometer (Bio-Rad, Hercules, USA).[27]

Western blot analysis of LC3-II and p62. Cells were seeded in 25-cm^2 cell culture flasks (3×10^5 for MDA-MB-231 and 5×10^5 for SK-BR-3) and treated with EPI, BAF, and EPI + BAF. Cells were centrifuged and then treated with RIPA lysis buffer (Beyotime) to obtain whole cell lysates. Then 30 μg protein from each group was separated by 15% SDS-PAGE and transferred to PVDF membranes. After blocking, PVDF membranes were incubated with primary antibodies against LC3, p62 (1:1000), and β-actin (1:2500) according to the manufacturer's recommendations. Membranes were then washed with Tris-buffered saline–Tween 20, and incubated with secondary antibodies for 1 h at room temperature. The bands were visualized and analyzed by a chemiluminescence detection system (Bio-Rad).

Measurement of autophagosomes and autolysosomes after mRFP-EGFP-LC3 transfection. Cells were seeded on coverslips in 6-well plates (1.0×10^6 for MDA-MB-231 and 1.2×10^6 for SK-BR-3). The plasmids of Mammalian expression of rat LC3 fused to monomeric Red Fluorescent Protein(mRFP) and Enhanced Green Fluorescent Protein(EGFP), also named ptfLC3 Plasmids, were transfected into cells with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated with EPI or EPI + BAF. The GFP and RFP fluorescence were measured under a fluorescent microscope (Nikon, Tokyo, Japan), and captured images of both GFP and RFP were merged. Red fluorescence indicated autolysosomes and yellow fluorescence, due to merging of both green and red fluorescence, indicated autophagosomes.[28,29]

Cell death assay using Trypan blue exclusion. MDA-MB-231 and SK-BR-3 cells were seeded at a density of 4×10^5 and 6×10^5, respectively, in 6-well plates and divided into seven groups, EPI, BAF, EPI + BAF, and control group, as described above, as well as Ac-DEVD-CHO, Ac-DEVD-CHO + EPI, and Ac-DEVD-CHO + EPI + BAF, which were treated with 20 μM Ac-DEVD-CHO for 48 h at 37°C. Cells were harvested by digestion and centrifugation then stained with Trypan blue. Dead cells were counted to estimate cell death.

4′,6′-Diamidino-2-phenylindole dihydrochloride staining. Cells were seeded on coverslips in 6-well plates and divided into control, BAF, EPI, and EPI + BAF treatment groups. Both attached and detached cells were collected and fixed with 4% paraformaldehyde for 10 min in 4°C. Fixed cells were then stained with DAPI for 15 min at 37°C in the dark, and fluorescence of DAPI was measured under a fluorescent microscope.

Propidium iodide staining and cell cycle assay for sub-G populations. Cells were seeded on coverslips in 6-well plates and divided into BAF, EPI, EPI + BAF, and control groups. After treatment cells were harvested by centrifugation, they were fixed with 75% ethanol for 24 h at −20°C. The cells were washed, stained with propidium iodide, and measured by flow cytometry (Beckman, Coulter, Pasadena, USA) at 488 nm.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay. The TUNEL assay was carried out using a One-Step TUNEL Apoptosis Assay Kit. Cells were seeded on coverslips in 6-well plates, and divided into control, BAF, EPI, and EPI + BAF treatment groups. Both attached and detached cells were collected and fixed with 4% paraformaldehyde for 1 h at room temperature, then were treated with 0.5% Triton X-100 for 15 min at room temperature. The TUNEL reaction mixture was prepared according to the manufacturer's instructions and applied to cells for 1 h at 37°C in the dark in a humidified atmosphere. We also used DAPI to stain nuclei simultaneously. The TUNEL-positive cells were counted under a fluorescence microscope.

Mitochondrial membrane potential assay. Cells were seeded on coverslips in 6-well plates and treated with or without EPI for 48 h, and incubated with 5 μg/mL rhodamine 123 for 10 min at 37°C. After incubation with rhodamine 123, cells were washed twice and incubated with fresh medium at 37°C for 1 h, and then incubated with PBS with 1 μg/mL DAPI at 37°C for 10 min. Cells were then observed under a fluorescence microscope.

Transmission electron microscopy. Both EPI-treated and control cells were fixed with a solution containing 3% glutaraldehyde in 0.1 M PBS (pH 7.3). The fixed cells were post-fixed with 1% osmic acid for 2 h then dehydrated in increasing concentrations (50% → 70% → 90% → 100%) of acetone, infiltrated with Epon-812 epoxy resin and pure acetone (1:1) solution for 30 min, and infiltrated with Epon-812 for 2 h. The samples were embedded in an Epon-812 embedding kit at 40°C for 12 h, then at 70°C for 24 h. Ultrathin sections were sliced using the Leica EM UC6 microtome (Leica, Wetzlar, Germany), then stained with uranyl acetate and lead citrate in a Leica EM stainer and examined using an H7650 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV. Digital images were obtained using the Hitachi TEM System.

Caspase-3 and caspase-9 activity assay. Cells were seeded in 6-well plates and divided into control, BAF, EPI, and EPI + BAF treatment groups. Thirty microliters of lysis buffer provided in the assay kit was added to resuspend the collected cells, followed by incubating on ice with light agitation for 30 min. The supernatants were collected after cells were centrifuged at 13 000 g for 10 min. Then 10 μL supernatant was used for measuring protein using Bradford reagent. Another 20 μL was used to assay caspase-3 and caspase-9 activity. Caspase-3 was assayed with Ac-DEVD-ρNA as substrate and caspase-9 with Ac-LEHD-ρNA. Both were incubated at 37°C for 4 h and OD values were read at 405 nm with a microplate reader.

Cytochrome c release measured with Western blot analysis. MDA-MB-231 cells (4×10^6) and SK-BR-3 cells (6×10^6) were seeded in 75-cm^2 cell culture flasks and treated with BAF, EPI, EPI + BAF, or control medium. Cells were collected, counted, and placed on ice for 30 min. Samples were then homogenized and cytoplasmic and mitochondrial protein lysates were separated by differential centrifugation (whole cell lysates was centrifuged at 1000*g* for 10 min to obtain the supernatant. The acquired supernatant was centrifuged at 3500*g* for 10 min and collected. Protein of each sample was measured with bicinchoninic acid reagent, and 50 μg protein was selected for Cyt C measurement using Western blot with GAPDH as an internal reference for cytoplasmic protein and COX-IV was the internal reference for mitochondrial protein. Anti-GAPDH was diluted to 1:2500, anti-COX-IV was diluted to 1:1500, and anti-Cyt C was diluted to 1:1000. Goat anti-mouse and goat anti-rabbit secondary antibodies were diluted to 1:10 000.

Effect of AC on autophagy, cytotoxicity, and apoptosis in EPI-treated breast cancer cells. A group of assays including MDC sequestration, Trypan blue, and caspase activity assay were carried out. In both MDC and caspase activity assays, cells were divided into four groups treated with control medium,

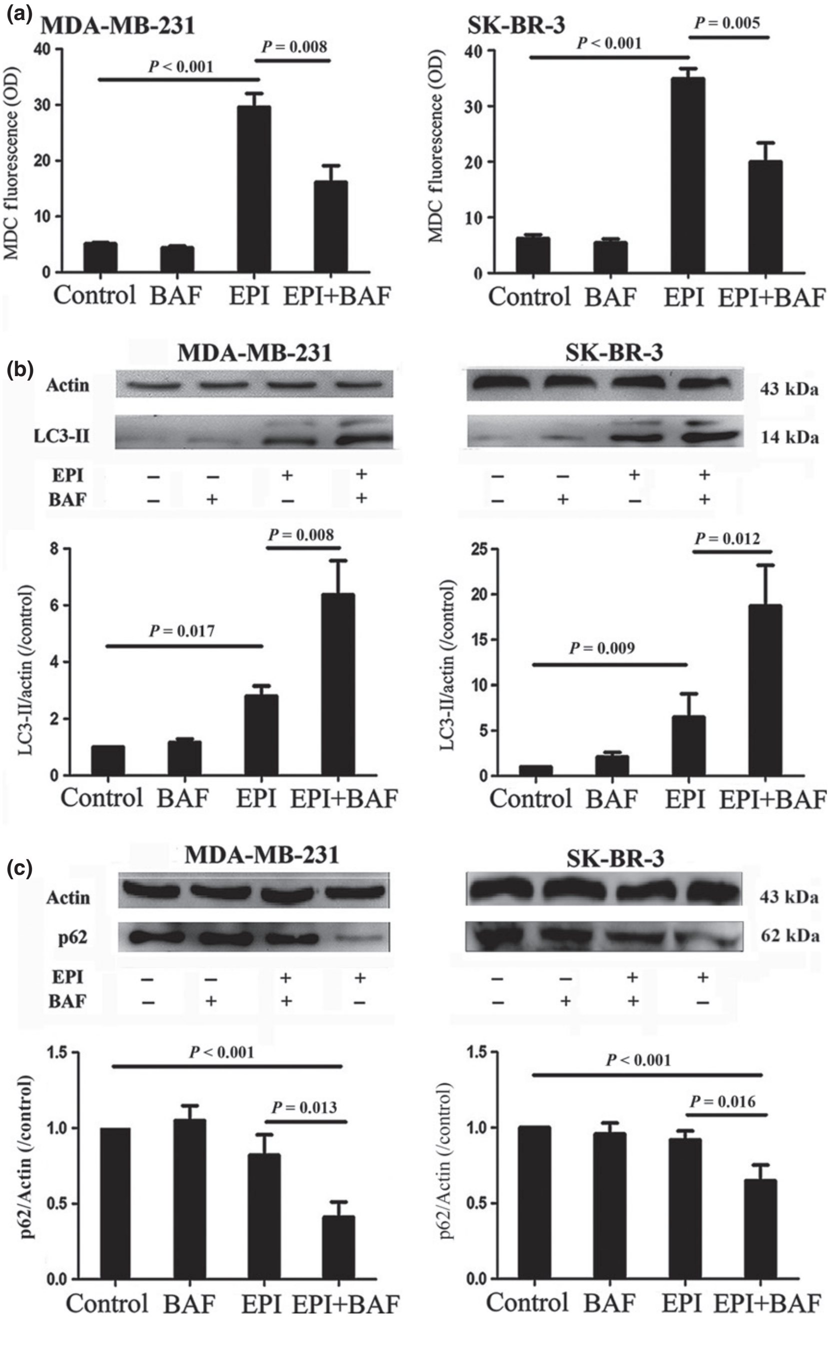

Fig. 1. Epirubicin (EPI) induced autophagy and bafilomycin A1 (BAF)'s effect on it. (a) MDA-MB-231 and SK-BR-3 cells were treated with BAF, EPI, or EPI + BAF and monodansylcadaverine (MDC) sequestration was measured. Optical density (OD) values are from three independent experiments. (b) Cells were treated with BAF, EPI, EPI+BAF and control, and microtubule-associated protein 1 light chain 3 form II (LC3-II) (14 kDa) expression was measured. Intensity values and LC3-II data are from three independent experiments. (c) Cells were treated as described and p62 (62 kDa) expression was measured. Intensity values and LC3-II data are from three independent experiments.

AC, EPI, or EPI + AC. In the Trypan blue assay, EPI + AC + Ac-DEVD-CHO was an additional group. For AC treatment, 10 mM AC was added to cells 24 h before harvesting. The MDC sequestration, Trypan blue, and caspase activity assays were carried out as described above.

Downregulation of Atg7 with siRNA. The si-Atg7 and control siRNA were transfected into MDA-MB-231 and SK-BR-3 by Lipofectamine 2000. The interference efficiencies of siRNAs were determined through Real-time qPCR and Western blot analysis. Real-time qPCR was undertaken with the primers. For Western blot analyses, each whole cell lysates sample was electrophoresed in 10% SDS-polyacrylamide gels, and then blotted onto PVDF membranes. Primary antibodies against Atg7 (1:1000) and β-actin (1:2000) were used following the manufacturer's recommendations. The results were visualized by the chemiluminescence detection system.

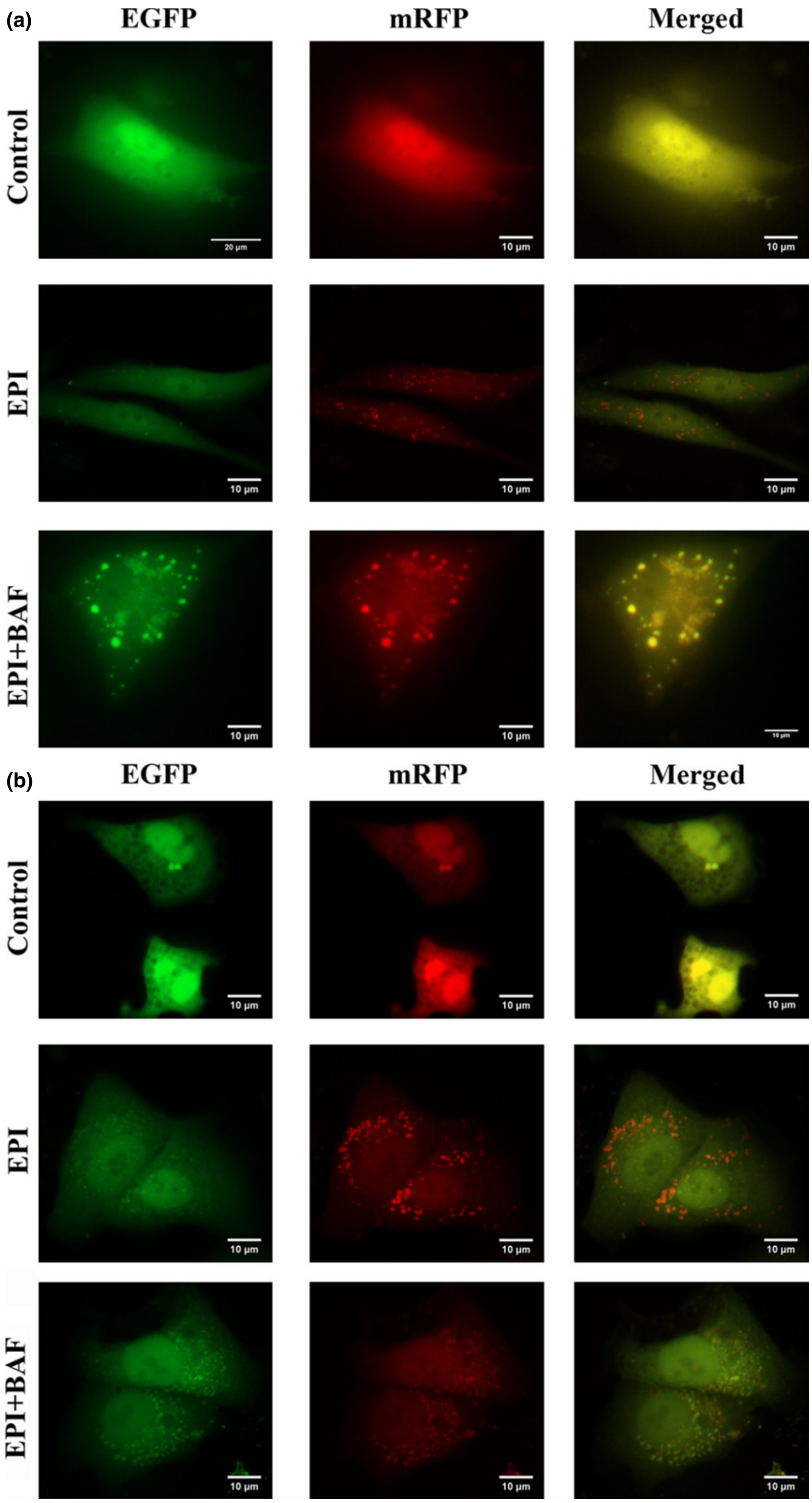

Fig. 2. Detection of autophagosomes and autolysosomes was carried out under fluorescence microscopy after transfection of breast cancer cell lines with monomeric red fluorescent protein (mRFP)–enhanced GFP (EGFP)–microtubule-associated protein 1 light chain 3. MDA-MB-231 (a) and SK-BR-3 (b) cells were transfected as described and 24 h later treated with control, EPI and EPI + BAF. GFP and RFP fluorescence were imaged under a microscope (×600).

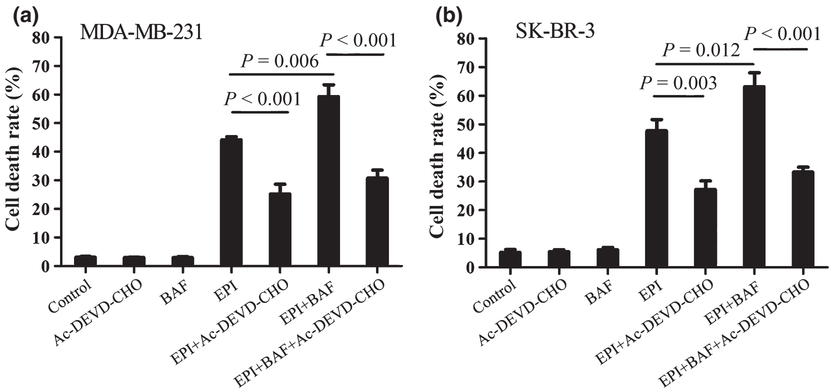

Fig. 3. Cell death in MDA-MB-231 (a) and SK-BR-3 (b) breast cancer cell lines after treatment with epirubicin (EPI) with or without bafilomycin A1 (BAF) and 50 μM Ac-DEVD-CHO for 48 h. Cell viability was measured with Trypan blue assay. Data were from three independent experiments.

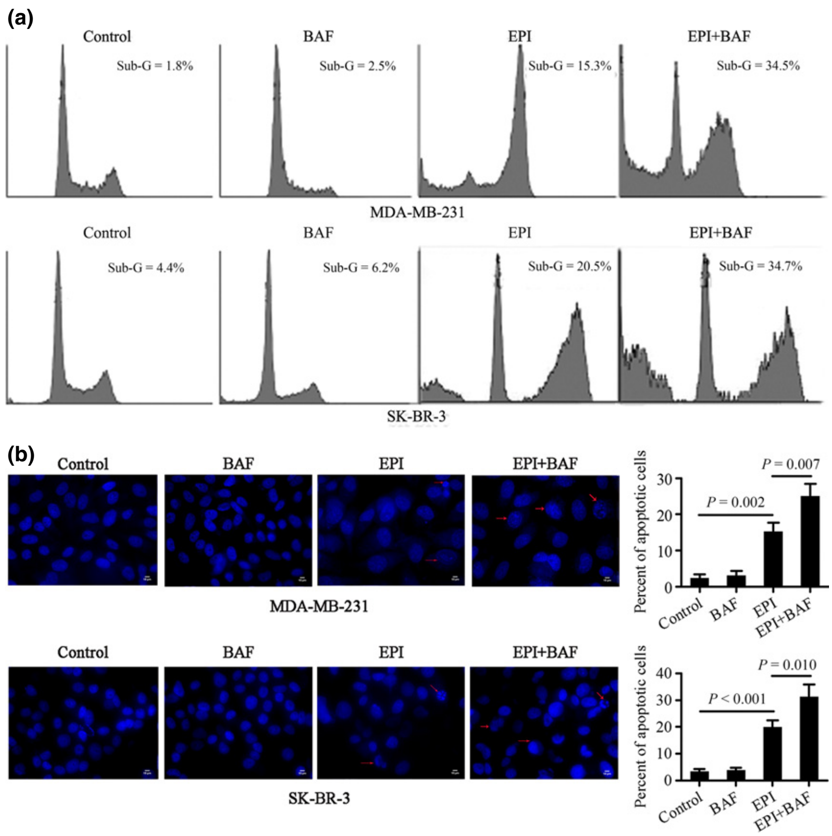

Fig. 4. Apoptosis assay in MDA-MB-231 and SK-BR-3 breast cancer cells after treatment epirubicin (EPI) with or without bafilomycin A1 (BAF). (a) Propidium iodide stained cells were assessed for sub-G_1 populations. (b) DAPI stained cells observed under a fluorescence microscope (×600) indicated apoptosis (apoptotic cells are indicated with red arrows). Data are from three independent experiments.

To study the effect of si-Atg7 on autophagy and apoptosis in EPI-treated cells, cells transfected with si-Atg7 or control siRNA and cells without transfection were collected, allowed to grow overnight, and divided into different groups. In both the MDC and caspase activity assay, the groups were siAtg7, EPI, EPI + siAtg7, and EPI + control siRNA. In the Trypan blue assay, the EPI + siAtg7 + Ac-DEVD-CHO-treated group was included with the five groups above. All of these assays were carried out as described above.

Statistical analyses. Data are from three independent experiments. Statistical comparisons of means were undertaken with ANOVA and $P \leq 0.05$ was considered statistically significant.

Results

Epirubicin induced autophagy in MDA-MB-231 and SK-BR-3 cells, and BAF inhibited EPI-induced autophagy by blocking autolysosome formation. Epirubicin treatment of both cell lines revealed an IC_{50} at 48 h of 3.0 μM in MDA-MB-231 cells and 2.0 μM in SK-BR-3 cells. Bafilomycin A1 (5, 10, and 20 nM) were not cytotoxic to either cell line at 24 h (Fig. S1), so 24 h of treatment with 20 nM BAF was chosen for experimental treatments.

The OD of MDCs for each treatment was measured and controls and BAF samples were not significantly different. Fluorescence of MDC in EPI-treated cells significantly increased in both cell lines compared to controls. Fluorescence after EPI + BAF treatment decreased compared to EPI treatment (Fig. 1a). Levels of LC3-II protein did not change after BAF treatment in either cell line, but increased after EPI treatment and increased further after EPI + BAF treatment (Fig. 1b). The p62 protein level significantly reduced after EPI treatment in both cell lines, whereas the combination of EPI and BAF reversed this trend. Bafilomycin A1 alone did not show any significant increase (Fig. 1c).

After cell transfection to assess autolysosome populations, measurement of fluorescence in controls revealed little or no fluorescence in either cell line. Treatment with EPI caused

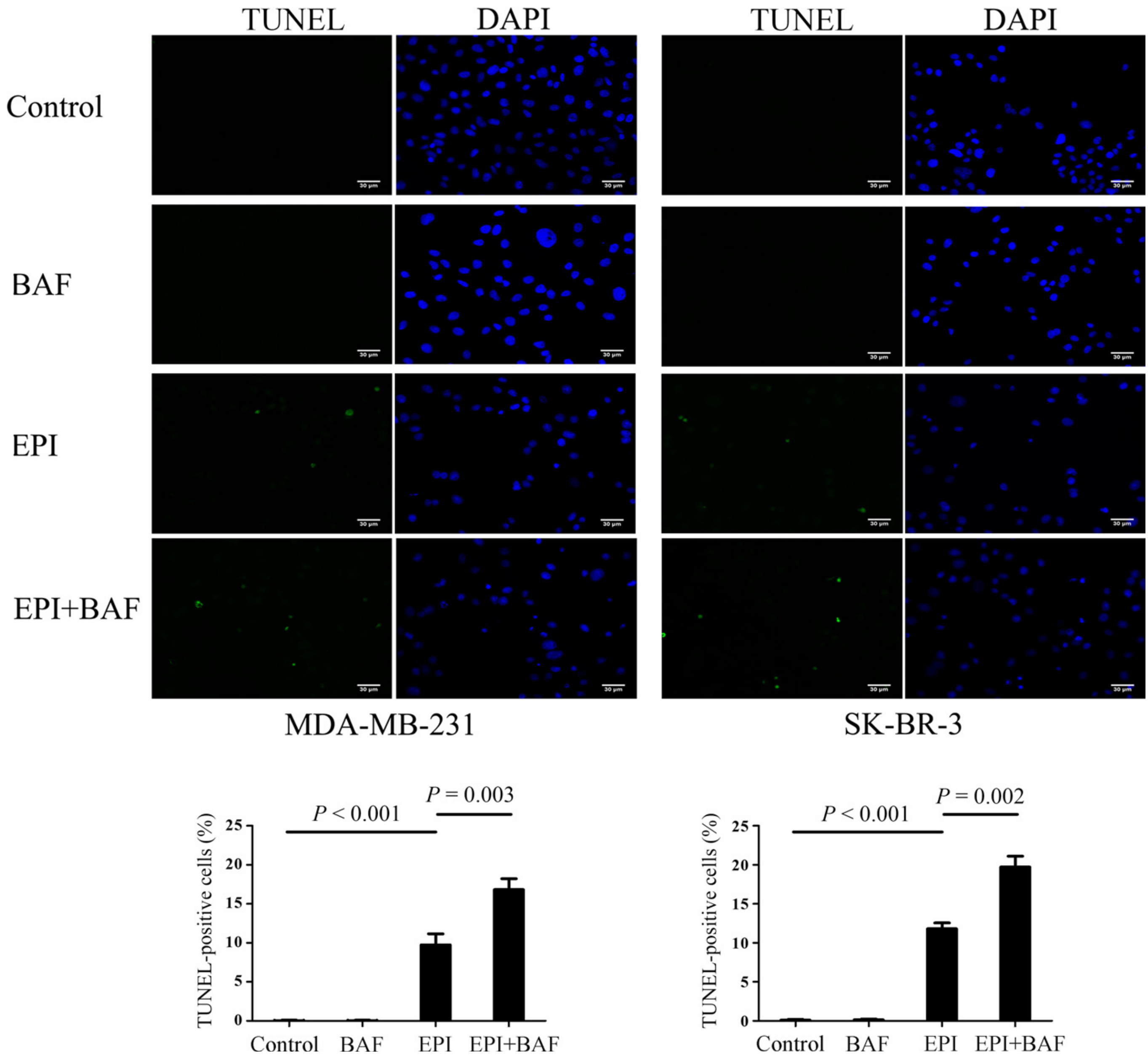

Fig. 5. Terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) staining of MDA-MB-231 and SK-BR-3 breast cancer cells treated with epirubicin (EPI) with or without bafilomycin A1 (BAF). Light green cells are TUNEL-positive staining cells. The percentage of TUNEL-positive cells was determined by counting cells in at least eight fields and more than 500 cells in total. Data were from three independent experiments.

greater red fluorescence of mRFP in both cell lines and this exceeded the green fluorescence of enhanced GFP. Merging images in a photofluorogram confirmed that most fluorescence was attributed to autolysosomes. In cells treated with EPI + BAF, both red and green fluorescence increased significantly, but in the merged photofluorogram, most fluorescence was attributed to autophagosomes that were not combined with lysosomes (Fig. 2).

According to a report by Mizushima and Yoshimori,[30] the increase of LC3-II indicates the increase of autophagosomes, which can be caused by either increased autophagic flux or inhibition of autolysosomes formation. Both p62 and p62-bound polyubiquitinated proteins become incorporated into the completed autophagosome and are degraded in autolysosomes, thus also serving as read-out of autophagic degradation. These results indicated that EPI induced autophagy in both cells, and BAF inhibited EPI-induced autophagy by inhibiting the formation of autolysosomes.

Epirubicin induced apoptosis in MDA-MB-231 and SK-BR-3 cells and blocking autophagy by BAF boosted EPI-triggered apoptosis. Trypan blue assay was used to assess cell death rate. We discovered that 24 h of treatment of 20 nM BAF was not cytotoxic to either cell line, nor was the caspase-3 inhibitor Ac-DEVD-CHO. However, Ac-DEVD-CHO decreased cell death induced by EPI in both of the cell lines, and EPI + BAF significantly increased cell death compared to EPI. In addition, Ac-DEVD-CHO treatment decreased cell death induced by EPI + BAF in both cell lines (Fig. 3).

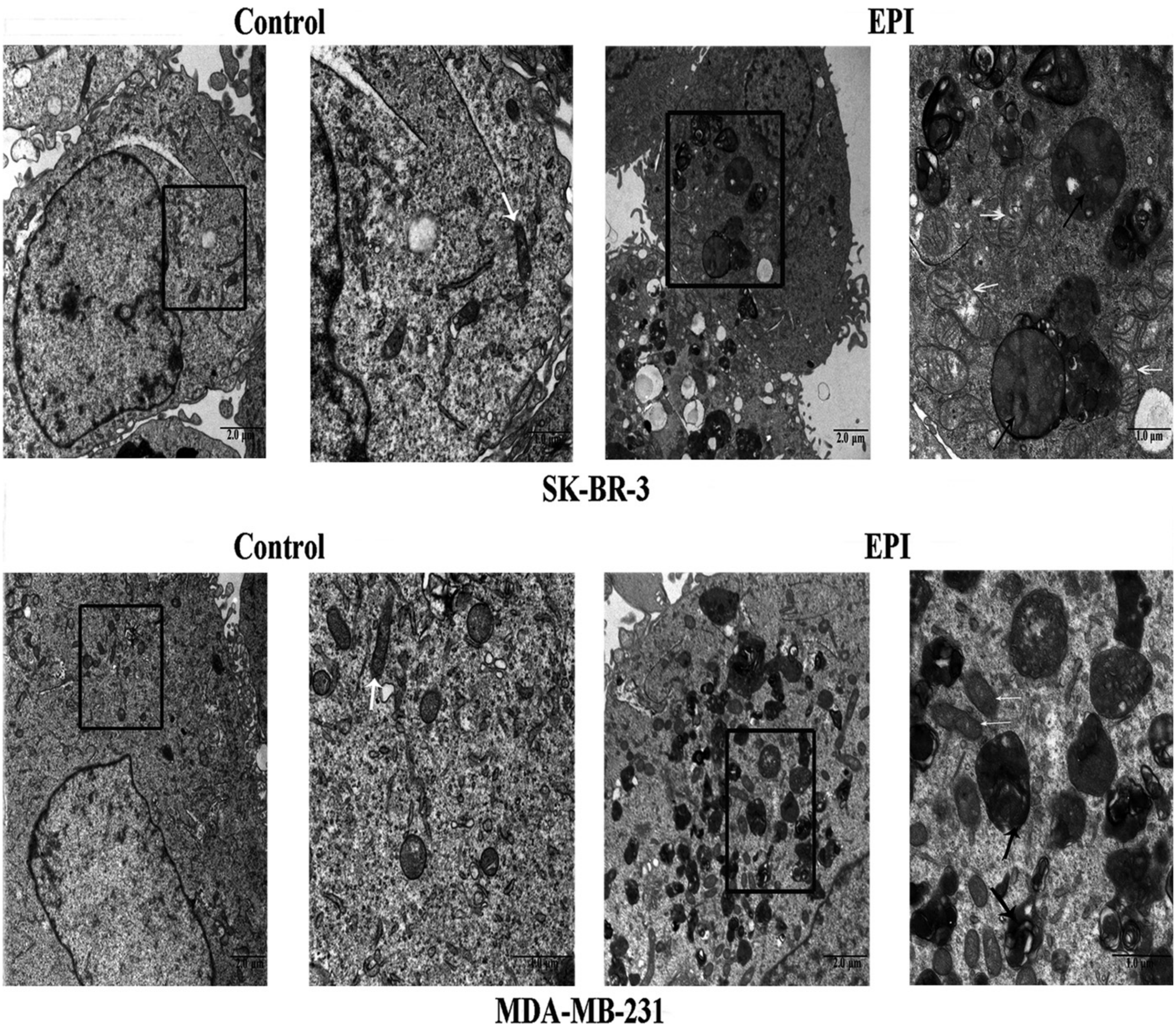

Fig. 6. Structures of mitochondria and autophagosomes were analyzed by electron microscopy (×15 000 and ×40 000) after cells were treated with or without epirubicin (EPI). In higher magnification images, the white arrows indicate the structures of mitochondria; the black arrows indicate the structures of autophagsomes that contain mitochondria-like structures.

Sub-G populations of cells were measured in both cell lines. Bafilomycin A1 treatment alone did not alter the number of sub-G cells in either cell line, but EPI significantly increased sub-G cells in G_2/M arrest. Treatment with EPI + BAF increased the number of sub-G cells more than EPI alone (Fig. 4a).

Normal cells showed intact nuclei and equally chromatin and apoptotic cells showed chromatin condensation, nuclei fragmentation, cell shrinkage, or even apoptotic body. These nuclear changes typical of apoptosis can be detect through DAPI staining.[31,32] Our results indicated that BAF did not cause apoptosis compared to controls, but EPI increased apoptotic cells in both cell lines and this was increased even more after EPI + BAF treatment (Fig. 4b). Staining with TUNEL also showed that apoptotic cells occurred under EPI treatment, and EPI + BAF enhanced this effect (Fig. 5).

These results indicated that EPI triggered apoptosis in both cell lines, and increased further under treatment with EPI + BAF.

Bafilomycin A1 enhanced EPI-triggered mitochondrial intrinsic apoptotic pathway. The MMP was lost in both cell lines after 48 h of treatment with EPI (Fig. S2). Electron microscopy was used to further identify the effect of EPI on mitochondria. The results showed that mitochondria became swollen in EPI-treated cells, and mitochondria-like structures could be observed in some autophagosomes (Fig. 6). It was suggested that MMP is damaged when MDA-MB-231 and SK-BR-3 cells are treated with EPI, but EPI-induced autophagy could engulf the damaged mitochondria in both cell lines.

In Western blot analysis, the increase of Cyt C in cytoplasm following a decrease in mitochondria indicates the release of Cyt C. Cytochrome C release was increased after EPI treatment and EPI + BAF increased Cyt C release further. Bafilomycin A1 treatment did not change Cyt C compared to control (Fig. 7a). Caspase-9 and -3 activities were measured with spectrophotometry. The activity of both caspases was increased after EPI treatment in both cell lines, and increased further

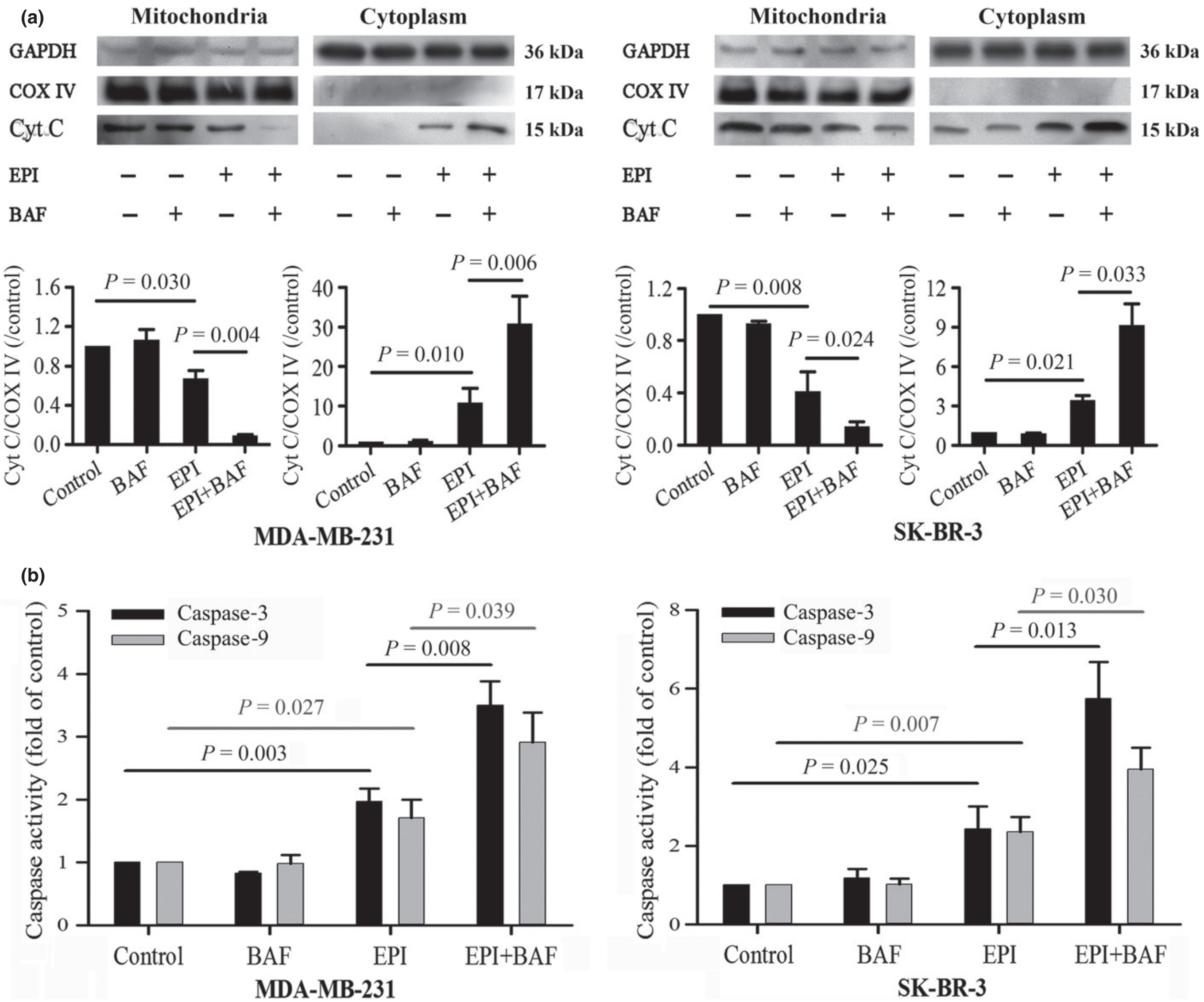

Fig. 7. Cytochrome C (Cyt C) release and caspase-3 and -9 activities were measured in MDA-MB-231 and SK-BR-3 breast cancer cells after treatment with control, BAF, EPI and EPI + BAF. (a) GAPDH was an internal control for cytosolic fractions and COX IV was a control for mitochondrial fractions. Intensity values and ratios of COX IV to controls are from three independent experiments. (b) MDA-MB-231 and SK-BR-3 cells were treated as depicted in Methods. Caspase-3 and -9 activities were assayed with Ac-DEVD-pNA and Ac-LEHD-pNA, respectively. Opitcal density values and fold-change data are from three independent experiments.

after EPI + BAF treatment. Bafilomycin A1 did not alter their activities (Fig. 7b).

The release of Cyt C from mitochondria to cytoplasm together with enhanced caspase-3 and -9 activities are consistent with the mechanism of mitochondrial intrinsic apoptosis, and these results suggested that EPI induced mitochondrial-dependent apoptosis in both cell lines; EPI + BAF further enhanced this trend in both cells, probably correlated with BAF's effect of inhibiting EPI-triggered autophagy as BAF alone could affect neither apoptosis nor autophagy.

Ammonium chloride inhibited autophagy and enhanced cytotoxicity of EPI in breast cancer cells with increasing caspase-3 and -9. The MDC sequestration assay showed that the OD value of EPI + AC significantly decreased compared with the EPI-treated group in both cell lines, whereas AC alone did not show such an effect compared with the control group (Fig. 8a). Trypan blue assay showed that AC enhanced EPI-induced cell death, whereas Ac-DEVD-CHO reduced the cell death rate compared with the EPI + AC-treated group (Fig. 8b). In caspase-3 and -9 activity assays, EPI + AC treatment significantly increased the activities of both caspase-3 and -9 compared with EPI treatment alone (Fig. 8c). These results suggest that, similar to BAF, AC also inhibits EPI-induced autophagy in breast cancer cells MDA-MB-231 and SK-BR-3, and increases cell death as well as caspase-9 and caspase-3-dependent apoptosis.

Inhibition of autophagy by si-ATG7 increased cytotoxicity of EPI in breast cancer cells. The Atg7 RT-qPCR analysis showed that the specific si-Atg7 downregulated Atg7 by 60–70% (Fig. 9a) compared with siRNA control. Western blot analysis also showed that Atg7 protein level significantly decreased in si-Atg7 transfected cells compared with cells transfected with control siRNA (Fig. 9b).

The results of MDC sequestration assay showed that the combination of EPI + si-Atg7 reduced the OD value compared

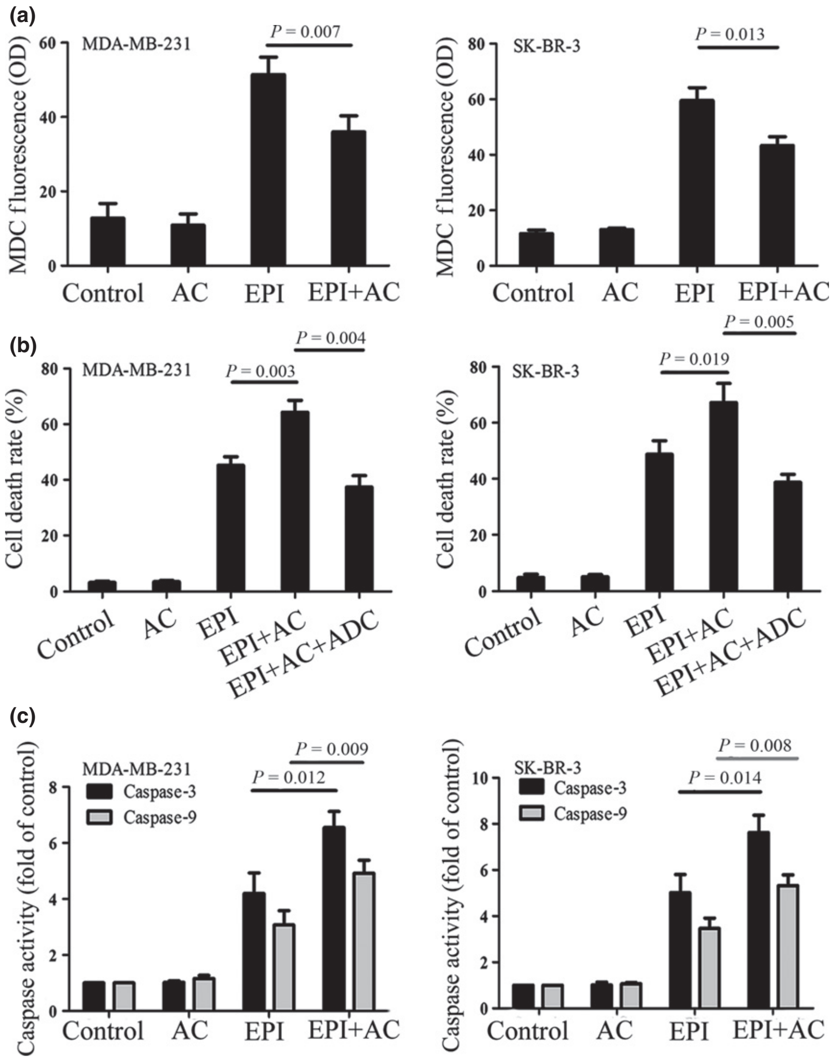

Fig. 8. Effect of ammonium chloride (AC) on autophagy and apoptosis in epirubicin (EPI)-treated MDA-MB-231 and SK-BR-3 breast cancer cells. (a) Cells were treated with EPI for 48 h with or without 10 mM AC (added 24 h before harvesting), then stained with 0.1 mM monodansylcadaverine (MDC) for 1 h before MDC sequestration was measured. Optical density (OD) values are from three independent experiments. (b) Cells were treated with EPI and 50 μM Ac-DEVD-CHO (ADC) for 48 h and/or 10 mM AC (added 24 h before harvesting), and cell viability was measured with Trypan blue assay. Data of cell death rate were from three independent experiments. (c) Cells were treated as indicated in (a), and caspase-3 and -9 activities were assayed with Ac-DEVD-pNA and Ac-LEHD-pNA, respectively. OD values and fold-change data are from three independent experiments.

with the EPI group (Fig. 9c). In addition, the death rate of cells treated with siATG7 + EPI was significantly boosted compared with EPI alone, and this trend can clearly be diminished by Ac-DEVD-CHO (Fig. 9d). Caspase-9 and -3 activities were also enhanced when combined with EPI and si-Atg7 (Fig. 9e). Control siRNA showed no such effect when combined with EPI. These results showed that Atg7 knockdown through siRNA inhibits autophagy, enhances apoptosis through caspase-9 and -3, and increases cytotoxicity in both cell lines treated with EPI.

Discussion

Previously we reported that MCF-7 cells treated with EPI did not undergo apoptosis but that autophagy was triggered, and blocking autophagy significantly induced apoptosis and increased toxicity of EPI. Thus, EPI-induced autophagy reduced apoptosis in MCF-7 cells.[21] Published reports indicate that different cytotoxic agents induce autophagy in MCF-7 cells with little apoptosis, and blocking autophagy dramatically increased apoptosis.[27,33] Different breast cancer cell lines may have different responses after treatment with the same agents: Choi's group reported that cordycepin-treated MCF-7 cells undergo autophagy-associated cell death with little apoptosis, but cordycepin-induced cell death in MDA-MB-231 cells is chiefly through apoptosis.[24] MCF-7 cells do not express caspase-3, a critical molecule in the apoptosis pathway.[25,26] Although MCF-7 cells can undergo apoptosis through a cascade reaction of caspase-6, -7, and -9,[34] or MDA-MB-231 cells may undergo caspase-3-independent apoptosis after treatment with other agents,[35] some other reports show that the lack of caspase-3 inhibits apoptosis in MCF-7 cells,[36,37] and specific knockdown of caspase-3 in MDA-MB-231 cells renders them resistant to cytotoxic agents.[38] Therefore, a caspase-3 defect may decrease apoptosis in MCF-7 cells treated with EPI, but MDA-MB-231 and SK-BR-3 cell lines may undergo apoptosis after EPI treatment. However, the effect of caspase-3 in controlling both apoptosis and autophagy requires further study. Interestingly, our results also show that EPI induced G_2/M arrest in both cell lines and these data were similar to previous work with EPI and MCF-7 cells.[39] Reports also suggest that EPI may not necessarily trigger autophagy in other breast cancer cells,[40] so how cells respond to EPI treatment is more complex than "either autophagy or apoptosis", and requires further study.

Some studies suggest that autophagy can promote cell death in breast cancer with a sufficient stimulus or when apoptosis is inhibited. In contrast, autophagy may be triggered by cytotoxic agents to prevent cell death and inhibitors of autophagy enhance cytotoxicity and increase cell death.[41] Our former

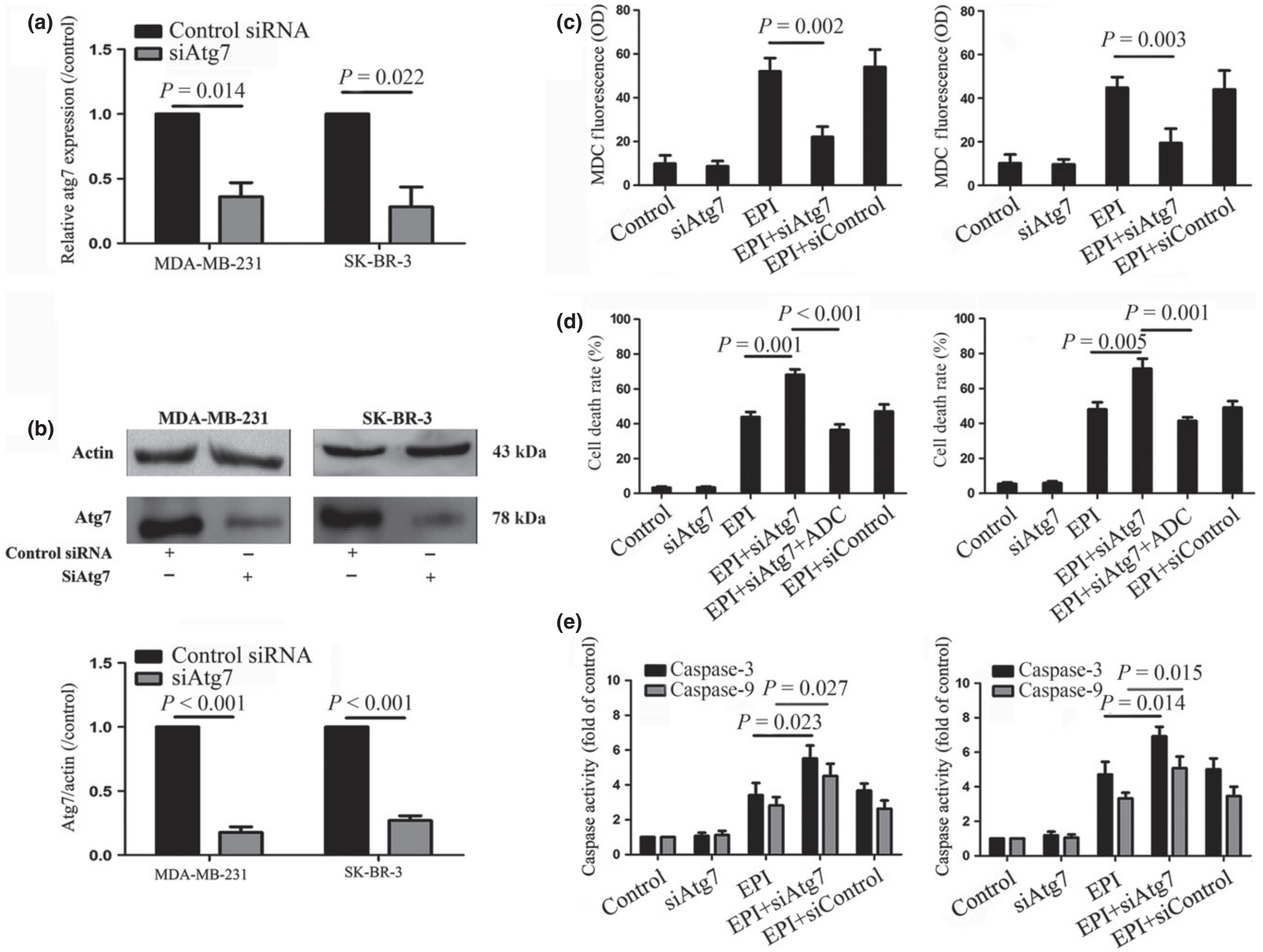

Fig. 9. Effect of Atg7 knockdown on autophagy and apoptosis in epirubicin (EPI)-treated MDA-MB-231 and SK-BR-3 breast cancer cells were measured after cells were transfected with siAtg7 or control siRNA. (a) Measurements of *Atg* mRNA expression by real-time quantitative PCR with β-*actin* as the reference gene after transfection. (b) Measurements of Atg7 protein level were carried out by Western blot after transfection. (c) Cells were treated with or without EPI, and stained with 0.1 mM monodansylcadaverine (MDC) for 1 h, and MDC sequestration was measured with a microplate fluorometer. (d) Cells were treated with or without EPI, and with or without Ac-DEVD-CHO (ADC) for 48 h, and the cell death rate was measured by Trypan blue assay. (e) Cells were treated with EPI for 48 h, and caspase-3 and -9 activities were assayed with Ac-DEVD-pNA and Ac-LEHD-pNA, respectively. All values and fold-change data are from three independent experiments.

study confirmed that blocking EPI-induced autophagy increased caspase-9-dependent intrinsic apoptosis and promoted cell death.[21] Here, we report that EPI triggered both apoptosis and autophagy in MDA-MB-231 and SK-BR-3 cells, and inhibited autophagy with BAF induced apoptosis and sensitized cells to EPI. These data agree with previous findings in MCF-7 cells and are consistent with a report that autophagy inhibition enhances the therapeutic response in both anthracycline-sensitive and -resistant MDA-MB-231 cells.[42] Another report shows that trastuzumab, an anti-human epidermal growth factor receptor 2 mAb, induced cytoprotective autophagy in SK-BR-3 cells.[43] In summary, autophagy induced by cytotoxic agents may either inhibit or promote survival in breast cancer cells, and the factors to decide between two opposite effects is worthy of further study.

How autophagy decreases cell death is unclear, but inhibition of intrinsic apoptosis may occur by autophagosomes consuming damaged mitochondria coupled with reduced Cyt C released in treated cells.[44–46] Cytochrome C release is necessary for intrinsic apoptosis because it cleaves/activates caspase-9 and, eventually, downstream caspase-3. Our former report showed that MMP was damaged in MCF-7 cells treated with EPI, but activation of caspase-9 did not occur.[21] This study indicates that most MDA-MB-231 and SK-BR-3 cells treated with EPI lost MMP and Cyt C release increased, as did activation of caspase-9 and -3. Inhibition of autophagy with BAF promoted this effect and increased apoptosis. Therefore, protective effects of autophagy in EPI-treated breast cancer cells may involve autophagic consumption of mitochondria and less Cyt C release to inhibit EPI-induced mitochondrial intrinsic apoptosis. Other studies indicate that different mechanisms contribute to cytoprotective autophagy induced by various cytotoxic agents in breast cancer cells, including reactive oxygen species and the inositol requiring enzyme 1 signaling pathway.[35,47,48] Autophagy may protect breast cancer cells through multiple mechanisms and these might be drug-specific; this also warrants further study.

In autophagy studies, both BAF and AC are often used as autophagy inhibiting agents.[29,49] Bafilomycin A1, from the bafilomycin family of toxic macrolide antibiotics, specifically inhibits the vacuolar-type H^+-ATPase, and thus prevents the maturation of autophagic vacuoles by inhibiting the fusion between autophagosomes and lysosomes, and ultimately inhibits autophagy.[11] Ammonia chloride is a weak base that could become protonated and accumulates inside lysosomes, elevating the pH and inactivating lysosomal hydrolases. This change in pH inhibits the fusion of autophagosomes with lysosomes.[50] In our study, we observed that both BAF and AC could inhibit EPI-induced autophagy in breast cancer cells, and could enhance apoptosis as well as cytotoxicity. Another approach to inhibit autophagy is through *Atg* gene knockdown. The Atg family of autophagy regulatory proteins regulate the formation of autophagosomes during the initiation of autophagy. This includes Atg7, which plays a predominant role in maturation of autophagic vacuoles by engaging other proteins to the autophagosomal membrane.[51] We found that in different breast cancer cell lines, downregulation of Atg7 significantly reversed EPI-triggered autophagy, and increased apoptosis as well as cell death.[21] Different autophagy inhibitors including BAF, AC, and si-Atg7 all sensitize breast cancer cells to EPI.

Thus, EPI induced both autophagy and apoptosis in MDA-MB-231 and SK-BR-3 cells and autophagy reduced cell death by engulfing damaged mitochondria and inhibiting mitochondrial intrinsic apoptosis. Autophagy inhibition through chemical agents like BAF and AC or knockdown of the *Atg* gene, such as *Atg7*, enhanced apoptosis and sensitized EPI-treated cells. Therefore, it is suggested that inhibition of autophagy may contribute to enhanced curative effects of EPI in breast cancer therapy. However, the molecular mechanism of autophagy and EPI-resistance in breast cancer cells still requires in-depth studies, and blockade of autophagy enhancing apoptosis and sensitizing breast cancer under treatment must be further verified by study *in vivo*.

Acknowledgments

This research was funded and supported by the Science and Technology Program of Sichuan Province (Grant No. 2011SZ0223). We thank LetPub for its linguistic assistance during the preparation of this manuscript.

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AC	ammonium chloride (NH_4Cl)
BAF	bafilomycin A1
Cyt C	cytochrome C
EPI	epirubicin
LC3-II	microtubule-associated protein 1 light chain 3 form II
MDC	monodansylcadaverine
MMP	mitochondrial membrane potential
mRFP	monomeric red fluorescent protein
OD	optical density
RT-qPCR	real-time quantitative PCR

References

1 Hortobagyi GN, de la Garza Salazar J, Pritchard K *et al.* The global breast cancer burden: variations in epidemiology and survival. *Clin Breast Cancer* 2005; **6**: 391–401.
2 NCCN Clinical Practice Guidelines in Oncology Breast Cancer. Version 3.2013, 2013.
3 Nielsen D, Maare C, Skovsgaard T. Cellular resistance to anthracyclines. *Gen Pharmacol* 1996; **27**: 251–5.
4 Gottesman MM. Mechanisms of cancer drug resistance. *Annu Rev Med* 2002; **53**: 615–27.
5 Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 2004; **64**: 463–77.
6 Kuma A, Mizushima N. Physiological role of autophagy as an intracellular recycling system: with an emphasis on nutrient metabolism. *Semin Cell Dev Biol* 2010; **21**: 683–90.
7 Mizushima N, Levine B. Autophagy in mammalian development and differentiation. *Nat Cell Biol* 2010; **12**: 823–30.
8 Teplova VV, Tonshin AA, Grigoriev PA, Saris N-EL, Salkinoja-Salonen MS. Bafilomycin A1 is a potassium ionophore that impairs mitochondrial functions. *J Bioenerg Biomembr* 2007; **39**: 321–9.
9 Shingu T, Fujiwara K, Bögler O *et al.* Inhibition of autophagy at a late stage enhances imatinib-induced cytotoxicity in human malignant glioma cells. *Int J Cancer* 2009; **124**: 1060–71.
10 Hernández-Breijo B, Monserrat J, Román ID *et al.* Azathioprine desensitizes liver cancer cells to insulin-like growth factor 1 and causes apoptosis when it is combined with bafilomycin A1. *Toxicol Appl Pharmacol* 2013; **272**: 568–78.
11 Bowman EJ, Sibers A, Altendorf K. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci USA* 1988; **1988**: 7972–6.
12 Yamamoto A, Yoshiro T, Tamotsu Y, Yoshinori M, Ryuichi M, Yutaka T. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting between autophagosomesand lysosomesin rat hepatomacell line, H-4-II-E cells. *Cell Struct Funct* 1998; **1998**: 33–42.
13 Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science* 2004; **306**: 990–5.
14 Cui Q, Tashiro S, Minami M, Ikejim T. Autophagy preceded apoptosis in oridonin-treated human breast cancer MCF-7 cells. *Biol Pharm Bull* 2007; **30**: 859–64.
15 Dragowska WH, Weppler SA, Wang JC *et al.* Induction of autophagy is an early response to gefitinib and a potential therapeutic target in breast cancer. *PLoS One* 2013; **8**: e76503.
16 Lisiak N, Paszel-Jaworska A, Bednarczyk-Cwynar B, Zaprutko L, Kaczmarek M, Rybczyńska M. Methyl 3-hydroxyimino-11-oxoolean-12-en-28-oate (HIMOXOL), a synthetic oleanolic acid derivative, induces both apoptosis and autophagy in MDA-MB-231 breast cancer cells. *Chem Biol Interact* 2014; **208**: 47–57.
17 Noureini SK, Esmaili H. Multiple mechanisms of cell death induced by chelidonine in MCF-7 breast cancer cell line. *Chem Biol Interact* 2014; **223**: 141–9.
18 Qadir MA, Kwok B, Dragowska WH, To KH, Le D, Bally MB. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Res Treat* 2008; **112**: 389–403.
19 Hassan C, Petra O, Mahmoud T, Rainer K, Gabriele M, Peter HR. Autophagy contributes to resistance of tumor cells to ionizing radiation. *Radiother Oncol* 2011; **99**: 287–92.
20 Singh P, Godbole M, Rao G *et al.* Inhibition of autophagy stimulate molecular iodine-induced apoptosis in hormone independent breast tumors. *Biochem Biophys Res Commun* 2011; **415**: 181–6.
21 Sun WL, Chen J, Wang YP, Zheng H. Autophagy protects breast cancer cells from epirubicin-induced apoptosis and facilitates epirubicin-resistance development. *Autophagy* 2011; **7**: 1035–44.
22 Moreno E, Doughty-Shenton D, Plano D *et al.* A dihydroselenoquinazoline inhibits S6 ribosomal protein signalling, induces apoptosis and inhibits autophagy in MCF-7 cells. *Eur J Pharm Sci* 2014; **63**: 87–95.
23 Arthur CR, Gupton JT, Kellogg GE *et al.* Autophagic cell death, polyploidy and senescence induced in breast tumor cells by the substituted pyrrole JG-03-14, a novel microtubule poison. *Biochem Pharmacol* 2007; **74**: 981–91.
24 Choi S, Lim MH, Kim KM, Jeon BH, Song WO, Kim TW. Cordycepin-induced apoptosis and autophagy in breast cancer cells are independent of the estrogen receptor. *Toxicol Appl Pharmacol* 2011; **257**: 165–73.
25 Kurokawa H, Nishio K, Fukumoto H, Tomonari A, Suzuki T, Saijo N. Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells. *Oncol Rep* 1999; **6**: 33–7.
26 Jänicke RU. MCF-7 breast carcinoma cells do not express caspase-3. *Breast Cancer Res Treat* 2009; **117**: 219–21.
27 Samaddar JS, Gaddy VT, Duplantier J *et al.* A role for macroautophagy in protection against 4-hydroxytamoxifen-induced cell death and the development of antiestrogen resistance. *Mol Cancer Ther* 2008; **7**: 2977–87.
28 Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 2007; **3**: 452–60.

29 Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell* 2010; **140**: 313–26.
30 Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy* 2007; **3**: 542–5.
31 Chen K, Zhang QY, Wang J *et al.* Taurine protects transformed rat retinal ganglion cells from hypoxia-induced apoptosis by preventing mitochondrial dysfunction. *Brain Res* 2009; **1279**: 131–8.
32 Tang Q, Li GW, Wei XN *et al.* Resveratrol-induced apoptosis is enhanced by inhibition of autophagy in esophageal squamous cell carcinoma. *Cancer Lett* 2013; **336**: 325–37.
33 Abedin MJ, Wang D, Mcdonnell MA, Lehmann U, Kelekar A. Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ* 2007; **14**: 500–10.
34 Liang Y, Yan C, Schor NF. Apoptosis in the absence of caspase 3. *Oncogene* 2001; **20**: 6570–8.
35 Wang Z, Li Y, Shi X *et al.* Blocking autophagy enhanced cytotoxicity induced by recombinant human arginase in triple-negative breast cancer cells. *Cell Death Dis* 2014; **5**: e1563.
36 Devarajan E, Sahin AA, Chen JS *et al.* Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. *Oncogene* 2002; **21**: 8843–51.
37 Essmann F, Engels IH, Totzke G, Schulze-Osthoff K, Jänicke RU. Apoptosis resistance of MCF-7 breast carcinoma cells to ionizing radiation is independent of p53 and cell cycle control but caused by the lack of caspase-3 and a caffeine-inhibitable event. *Cancer Res* 2004; **64**: 7065–72.
38 Yang SH, Zhou Q, Yang XH. Caspase-3 status is a determinant of the differential responses to genistein between MDA-MB-231 and MCF-7 breast cancer cells. *Biochim Biophys Acta* 2007; **1773**: 903–11.
39 Azab SS, El-Demardash E, Abdel-Naim AB, Youssef E, El-Sharkawy N, Osman AM. Modulation of epirubicin cytotoxicity by tamoxifen in human breast cancer cell lines. *Biochem Pharmacol* 2005; **70**: 725–32.
40 Shen M, Duan WM, Wu MY *et al.* Participation of autophagy in the cytotoxicity against breast cancer cells by cisplatin. *Oncol Rep* 2015; **34** (1): 359–67.
41 Esteve JM, Knecht E. Mechanisms of autophagy and apoptosis: recent developments in breast cancer cells. *World J Biol Chem* 2011; **2**: 232–8.
42 Chittaranjan S, Bortnik S, Dragowsa H *et al.* Autophagy inhibition augments the anticancer effects of epirubicin treatment in anthracycline-sensitive and -resistant triple-negative breast cancer. *Clin Cancer Res* 2014; **20**: 3159–73.
43 Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. *PLoS One* 2009; **4**: e6251.
44 Kundu M, Thompson CB. Macroautophagy versus mitochondrial autophagy: a question of fate? *Cell Death Differ* 2005; **12** (Suppl 2): 1484–9.
45 Ravikumar B, Berger Z, Vacher C, Okane CJ, Rubinsztein DC. Rapamycin pre-treatment protects against apoptosis. *Hum Mol Genet* 2006; **15**: 1209–16.
46 Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. *Neuroscience* 2009; **164**: 541–51.
47 Ozkan A, Ayhan A, Fiskin K. Combined effect of epirubicin and lymphokine-activated killer cells on the resistant human breast cancer cells. *Cell Biol Toxicol* 2004; **20**: 261–71.
48 Xu WH, Liu ZB, Hou YF, Hong Q, Hu DL, Shao ZM. Inhibition of autophagy enhances the cytotoxic effect of PA-MSHA in breast cancer. *BMC Cancer* 2014; **14**: 273.
49 Daniel K, Abdalla F, Abeliovich H. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012; **8**: 1–100.
50 Paludan C, Schmid D, Landthaler M *et al.* Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 2005; **307**: 593–6.
51 Ohsumi Y, Mizushima N. Two ubiquitin-like conjugation systems essential for autophagy. *Semin Cell Dev Biol* 2004; **15**: 231–6.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Cell viability measured in SK-BR-3 (a) and MDA-MB-231 (b) cells treated with bafilomycin A1 (BAF) for 0, 24, 48, and 72 h. Results are from three independent experiments.

Fig. S2. Mitochondria membrane potential (MMP) was detected by rhodamine 123 (Rh123) and DAPI after cells were treated with epirubicin (EPI). The fluorescence of Rh123 and DAPI was observed by fluorescence microscope (×200).