Received: 7 March 2018 | Revised: 8 April 2018 | Accepted: 11 April 2018

DOI: 10.1111/cas.13616

ORIGINAL ARTICLE

WILEY Cancer Science

Endoplasmic reticulum stress-mediated autophagy protects against β,β-dimethylacrylshikonin-induced apoptosis in lung adenocarcinoma cells

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Funding information
National Natural Science Foundation of China (Grant/Award Number: '81403143'), The Excellent Youth Talent Program of Zhejiang Provincial Hospital of Traditional Chinese Medicine (Grant/Award Number: '2D01417').

β,β-Dimethylacrylshikonin (DMAS) is an anti-cancer compound extracted from the roots of *Lithospermum erythrorhizon*. The present study aims to investigate the effects of DMAS on human lung adenocarcinoma cells in vitro and explore the mechanisms of its anti-cancer action. We showed that DMAS markedly inhibited cell viability in a dose- and time-dependent way, and induced apoptosis as well as autophagy in human lung adenocarcinoma cells. Furthermore, we found that DMAS stimulated endoplasmic reticulum stress and mediated autophagy through the PERK-eIF2α-ATF4-CHOP and IRE1-TRAF2-JNK axes of the unfolded protein response in human lung adenocarcinoma cells. We also showed that the autophagy induced by DMAS played a prosurvival role in human lung adenocarcinoma cells and attenuated the apoptotic cascade. Collectively, combined treatment of DMAS and pharmacological autophagy inhibitors could offer an effective therapeutic strategy for lung adenocarcinoma treatment.

KEYWORDS
apoptosis, autophagy, endoplasmic reticulum stress, lung adenocarcinoma, β,β-dimethylacrylshikonin

1 | INTRODUCTION

Lung cancer is one of the most frequently diagnosed cancers and the leading cause of cancer-related death worldwide.[1] Among all cases of primary lung tumors, lung adenocarcinoma is the predominant subgroup (30%-40%).[2] Chemotherapy plays a critical role in curing or controlling lung adenocarcinoma. However, as most widely used chemotherapeutic agents show severe side-effects and accompanying drug resistance, seeking novel and safe drugs for this devastating disease is an urgent need.

Natural products have been the most prolific source of new lead compounds for pharmaceutical development. Many anti-cancer drugs, such as etoposide, paclitaxel, and vincristine, are plant-derived and play crucial roles in chemotherapy.[3-5] β,β-Dimethylacrylshikonin (DMAS), one of the shikonin derivatives, is a typical component of naphthoquinone pigments extracted from the traditional medical herb *Lithospermum erythrorhizon* (Figure 1A).[6] To date, mounting evidence has shown that DMAS presents remarkable anti-tumor activity both in vitro and in vivo. For instance, DMAS suppresses Notch-1 activity, thereby inhibiting tumor growth in murine gastric cancer cell line xenografts.[7] Moreover, DMAS triggers apoptosis through a classic caspase-dependent pathway in human hepatocellular carcinoma cells.[8] In addition, DMAS also induces mitochondria-dependent apoptosis in human gastric cancer cells through mediation of the ERK1/2 signaling pathway.[9]

However, whether DMAS induces autophagy, and the role it plays in cell death in human lung adenocarcinoma cells, remain elusive. Autophagy is an evolutionarily conserved cellular self-

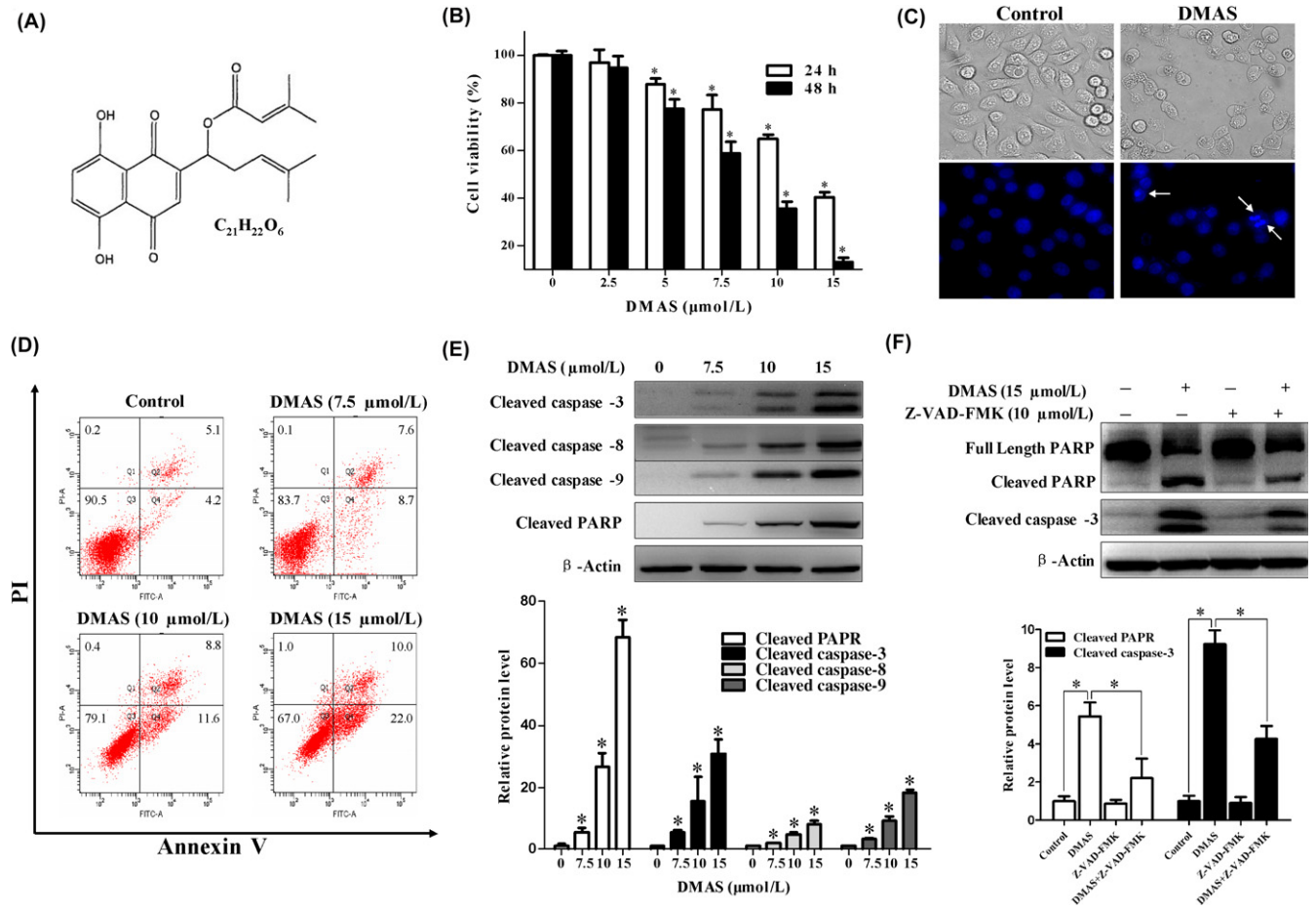


FIGURE 1 β,β-Dimethylacrylshikonin (DMAS) inhibits cell viability and induces apoptosis in human lung adenocarcinoma cells. A, Chemical structure of DMAS. B, PC-9 cells were treated with DMAS for 24 and 48 h, and then cell viability was evaluated by MTT assay. C, PC-9 cells were treated with DMAS (15 μmol/L) for 24 h and then stained with DAPI solution. Morphological and nuclear changes were assessed by fluorescence microscopy. White arrows indicate condensed nuclei. D, After treatment with DMAS for 24 h, PC-9 cells were stained with Annexin V-FITC/propidium iodide (PI) and detected by flow cytometry analysis. E, PC-9 cells were treated with DMAS for 24 h. Expression levels of indicated proteins were detected by western blot assay. F, PC-9 cells were incubated with DMAS for 24 h with or without pretreatment with Z-VAD-FMK (10 μmol/L). Expression levels of indicated proteins were studied by western blot assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group. PARP, poly ADP ribose polymerase

catabolic process, responsible for degrading and recycling unnecessary or damaged cytoplasmic components in a lysosome-dependent method for cell survival.[10] The process of autophagy begins with sequestering unnecessary byproducts or damaged organelles into autophagosomes that then fuse with lysosomes to form autolysosomes for degradation of inner cargoes.[11] Previous research suggested that numerous compounds could induce autophagy for cell survival or cell death by a variety of mechanisms (eg, salinomycin-induced cytoprotective autophagy in human lung cancer cells through the activation of endoplasmic reticulum stress, whereas isocryptotanshinone increased autophagic cell death in A549 lung cancer cells by inhibition of STAT3 activity).[12,13]

In the current study, the effects of DMAS on human lung adenocarcinoma cells in terms of cell viability, apoptosis, and autophagy were evaluated. Furthermore, the potential mechanisms involved in DMAS-induced apoptosis and autophagy were also investigated.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

DMAS (purchased from Tokyo Chemical Industry) was dissolved in DMSO, and the DMSO content in all groups was 0.1%. MTT, DAPI and SP600125 were obtained from Calbiochem (San Diego, CA, USA). RPMI 1640 medium and FBS were obtained from Gibco Life Technologies (Grand Island, NY, USA). Pan-caspase inhibitor (Z-VAD-FMK) was purchased from Beyotime Biotechnology Corporation (Shanghai, China). 3-Methyladenine (3-MA), chloroquine (CQ), 4-phenylbutyrate (4-PBA), and monodansylcadaverine (MDC) were obtained from Sigma-Aldrich (St Louis, MO, USA). Bafilomycin A1

was obtained from Selleck Chemicals (Houston, TX, USA). Primary antibodies (ie poly ADP ribose polymerase [PARP], cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, LC3B, Atg5, Beclin-1, Bip, phospho-eIF2α, ATF4, CHOP, IRE1α, and phosphor-JNK) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

2.2 | Cell culture

PC-9 and A549 cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were grown in a 5% CO_2 incubator at 37°C and cultured in RPMI 1640 medium with 10% (v/v) FBS.

2.3 | Cell viability assay

Effects of indicated agents on cell viability were studied through the MTT assay as described previously.[9]

2.4 | Nuclear staining with DAPI

After DMAS treatment, adherent cells were fixed for 30 minutes with cold acetone and permeabilized for 10 minutes with 0.1% Triton X-100 in PBS. After washing with PBS, cells were stained with DAPI in PBS at the concentration of 1 mg/mL for 30 minutes at room temperature in the dark. Cells were then washed with PBS and visualized using a fluorescence microscope (Leica DM4000; Leica, Wetzlar, Germany).

2.5 | Annexin V-FITC/PI staining assay

After treatment with the indicated methods, cells were trypsinized, rinsed and harvested. Apoptotic cells were detected by using an Annexin V-FITC/propidium iodide (PI) assay kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. A total of 10 000 cells were harvested and analyzed by using a flow cytometer (FACS-Canto II; Becton Dickinson, Franklin Lakes, NJ, USA).

2.6 | Monodansylcadaverine staining

Pretreated cells were washed with PBS, and incubated with a 50 μmol/L final concentration of MDC in PBS for 30 minutes at 37°C in the dark. After washing with PBS, the stained cells were fixed with 4% paraformaldehyde and then imaged immediately under a fluorescent microscope (Leica DM4000; Leica).

2.7 | Transmission electron microscopy

After treatment with indicated concentrations of DMAS for 24 hours, cells were collected by trypsinization, washed and fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, then post-fixed in 1% phosphate-buffered osmium tetroxide for 1 hour, dehydrated in graded acetone and ethanol, and embedded in epoxy resin. Ultrathin sections (80-90 nm) were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, then examined and photographed using an Olympus transmission electron microscope (TEM; Tecnai, Tokyo, Japan).

2.8 | Small interfering RNA transfection

Specific target sequences of Atg5 (sense 5′-GACUUACCGGACCACUGAAATT-3′, antisense 5′-UUCAGUGGUCCGGUAAGUCTT-3′), ATF4 (sense 5′-GCCUAGGUCUCUUAGAUGATT-3′, antisense 5′-UCAUCUAAGAGACCUAGGCTT-3′), CHOP (sense 5′-GUUUCCUGGUUCUCCCUUGGUCUUTT-3′, antisense 5′-AAGACCAAGGGAGAACCAGGAAACTT-3′), and the scrambled siRNA (5′-UUCUCCGAACG UGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGGAGAATT-3′) were synthesized by TranSheepBio-Tech (Shanghai, China). Cells were seeded into 6-cell plates overnight, and then transfected with specific siRNA by using a Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Cells were then treated with DMAS.

2.9 | Immunofluorescence staining

Cells were transfected with lentivirus Stub-RFP-Sens-GFP-LC3 (GeneChem, Shanghai, China) in strict accordance with the manufacturer's instructions. The cells were then treated with the indicated conditions. Expression of Stub-RFP and Sens-GFP was visualized and imaged by a fluorescence microscopy (Leica DM4000; Leica).

2.10 | Western blot assay

After treatment with the indicated conditions, cells were harvested, and lysed with RIPA buffer containing proteinase inhibitor cocktail (Calbiochem). Protein concentrations of cell lysates were determined with the BCA™ protein assay kit (Pierce, Rockford, IL, USA), followed by boiling in gel-loading buffer for 10 minutes at 100°C. Equal amounts of proteins were loaded and separated by SDS-PAGE and subsequently transferred to PVDF membrane (Millipore, Temecula, CA, USA). Following transfer, the membranes were blocked in TBST (TBS containing 0.1% Tween 20) containing 5% non-fat dried milk at room temperature for 2 hours, followed by incubation with appropriate primary antibodies overnight at 4°C. After washing with TBST 3 times for 10 minutes each, the membranes were incubated with HRP-conjugated appropriate secondary antibodies for 2 hours at room temperature. Specific protein bands were visualized using an enhanced chemiluminescence detection system (Immun-Star WesternC Kit; Bio-Rad, Hercules, CA, USA).

2.11 | Statistical analysis

All experiments were repeated at least 3 times. Mean ± SD was calculated in each group. Statistical analysis was carried out with 1-way analysis of variance (ANOVA). Differences were considered statistically significant at $^*P < .05$.

3 | RESULTS

3.1 | DMAS inhibits growth and induces apoptosis in human lung adenocarcinoma cells

To investigate the cytotoxic effect of DMAS on human lung adenocarcinoma cells, PC-9 cells were exposed to increasing concentrations of DMAS for various time points, and cell viability was subsequently evaluated by MTT assay. As shown in Figure 1B, DMAS significantly reduced the viability of PC-9 cells in both dose-dependent and time-dependent ways. Median inhibitory concentration (IC_{50}) of DMAS for PC-9 cells was 13.07 and 8.39 μmol/L at 24 and 48 hours, respectively.

To explore the underlying mechanism of DMAS-induced cell death, the apoptotic effect of DMAS in PC-9 cells was determined by various methods. First, morphological changes in DMAS-treated cells were observed under phase contrast microscope; PC-9 cells in the control group were in a typical polygonal or spindle-like intact appearance whereas DMAS-treated PC-9 cells showed cell shrinkage, rounding, and finally detached and floated in the culture medium (Figure 1C). Next, DAPI staining of the nuclei was carried out. DMAS induced nuclear condensation and apoptotic body formation in PC-9 cells compared to the negative control group (Figure 1C). In addition, flow cytometry was carried out using Annexin V-FITC/PI double staining to label PC-9 cells undergoing apoptosis, and DMAS was found to induce apoptosis in PC-9 cells in a concentration-dependent method (Figure 1D). Taken together, these results indicated that DMAS promoted PC-9 cell death through inducing apoptosis.

Caspase activation is considered a hallmark of the early stages of apoptosis.[14] To examine whether DMAS-induced apoptosis is associated with caspase activation, cleavage of caspase-3, caspase-8, caspase-9, and PARP was detected using western blot assay. We found that exposure of PC-9 cells to DMAS resulted in a dose-dependent increase in the cleavage of caspase-3, caspase-8, and caspase-9 (Figure 1E). Cleaved PARP, which is a substrate of caspase-3 and well-known apoptotic hallmark, was also increased with increasing concentrations of DMAS (Figure 1E). To further confirm the role of caspase activation in DMAS-induced apoptosis, we pretreated PC-9 cells with the pan-caspase inhibitor Z-VAD-FMK (10 μmol/L) prior to DMAS treatment. DMAS-induced cleavage of caspase-3 and PARP was almost completely abolished by pretreatment with Z-VAD-FMK (Figure 1F). In light of these results, DMAS could induce apoptosis in human lung adenocarcinoma PC-9 cells through the caspase-dependent apoptotic pathway. Similarly, our previous study has also shown that DMAS induces apoptosis in a caspase-dependent method in human lung adenocarcinoma A549 cells.[15]

3.2 | DMAS stimulates autophagy in human lung adenocarcinoma cells

To determine whether DMAS affects autophagy in human lung adenocarcinoma cells, several autophagic assays were conducted. Initially, MDC staining was carried out and viewed under a fluorescence microscope. MDC is an autofluorescent base which selectively accumulates in acidic vesicular organelles and, therefore, is used as a specific marker for autophagolysosomes.[16,17] As shown in Figure 2A, exposure of PC-9 and A549 cells to DMAS for 24 hours caused the accumulation of MDC-labeled vacuoles in the cytoplasm. Next, intracellular morphological changes of PC-9 and A549 cells were examined by use of TEM. Apparently, abundant autophagic vacuoles, which contain subcellular materials, were observed in PC-9 and A549 cells by DMAS treatment (Figure 2B). Furthermore, protein expression level of LC3B-II, a hallmark of autophagy,[18] was detected through the western blot assay. After treatment with DMAS (15 μmol/L) at the indicated times or with DMAS at the indicated concentrations for 24 hours, LC3B-II level increased in PC-9 and A549 cells in a dose- and time-dependent way (Figure 2C,D). We also measured the expression of autophagy-related proteins, Beclin-1 and Atg5. Expression levels of Beclin-1 and Atg5 increased in a concentration-dependent method (Figure 2D). Finally, we monitored the formation of autophagosomes in PC-9 and A549 cells stably transfected with tandem Stub-RFP-Sens-GFP-LC3. At the early stage of autophagy, autophagosomes display both red signals (Stub-RFP) and green signals (Sens-GFP). Autolysosomes develop red fluorescence only, as the Sens-GFP signal is sensitive to the acidic environment in the lysosome whereas the Stub-RFP signal is relatively stable. After PC-9 and A549 cell treatment with DMAS, autophagosomes were detected by fluorescence microscopy. Number of yellow (merging of green Sens-GFP signal and red Stub-RFP signal) puncta significantly increased in both PC-9 and A549 cells treated with DMAS compared with the negative control group (Figure 2E). The aforementioned results suggested that DMAS could induce autophagy in human lung adenocarcinoma cells.

Because autophagy is considered to be a dynamic and multistep process, we continued to monitor the autophagic flux induced by DMAS. As shown in Figure 3A, knockdown of Atg5 by siRNA decreased DMAS-induced LC3B-II formation in PC-9 and A549 cells. Furthermore, we cotreated PC-9 and A549 cells with DMAS (15 μmol/L) and autophagy inhibitors at different concentrations. Results showed that coincubation with DMAS and 3-MA (5 mmol/L), which blocks autophagy at the early stages, reduced LC3B-II formation in comparison to treatment with DMAS alone (Figure 3B). In contrast, combined treatment of DMAS and bafilomycin A1 (20 nmol/L) or chloroquine (CQ; 3 μmol/L), both of which block autophagy at the late stages, increased LC3B-II accumulation more than treatment with DMAS alone (Figure 3C,D). Collectively, these data further confirmed that DMAS could induce autophagy activation in human lung adenocarcinoma cells.

3.3 | DMAS induces autophagy through activation of endoplasmic reticulum stress in human lung adenocarcinoma cells

It is becoming increasingly clear that endoplasmic reticulum (ER) stress is one of the signaling pathways involved in regulation of

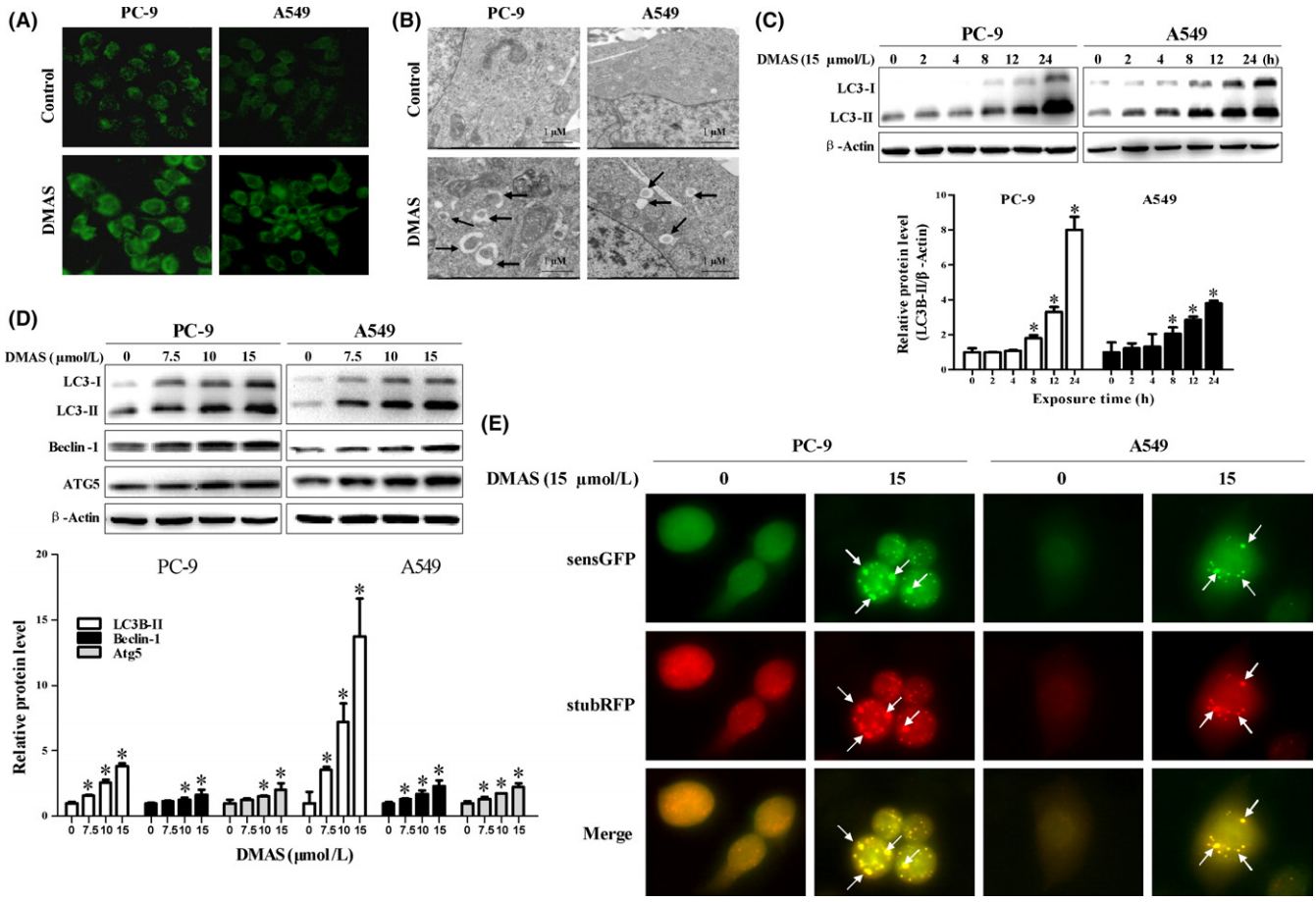


FIGURE 2 β,β-Dimethylacrylshikonin (DMAS) induces autophagy in human lung adenocarcinoma cells. A, PC-9 and A549 cells were treated with DMAS (15 μmol/L) for 24 h and then stained with MDC. Acidic vesicles were observed under a fluorescence microscope. B, PC-9 and A549 cells were treated with DMAS (15 μmol/L) for 24 h, then transmission electron microscopy (TEM) was carried out. Black arrows show membrane-bound vacuoles characteristic of autophagosomes. C, PC-9 and A549 cells were treated with DMAS (15 μmol/L) for the indicated times. Expression levels of indicated proteins were analyzed by western blot assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group. D, PC-9 and A549 cells were exposed to DMAS for 24 h. Expression levels of indicated proteins were detected by western blot assay. E, PC-9 and A549 cells were transfected with lentivirus Stub-RFP-Sens-GFP-LC3 and subsequently treated with DMAS (15 μmol/L) for 24 h. Fluorescent images were obtained by fluorescence microscopy and typical images are presented. White arrows indicate autophagosomes

autophagy.[19-21] ER stress activates the unfolded protein response (UPR), and the activation of the PERK-eIF2α-ATF4-CHOP axis and IRE1-TRAF2-JNK axes of the UPR is reported to be important for the mediation of ER stress-induced autophagy.[22] To determine whether ER stress was triggered by DMAS in PC-9 and A549 cells, we directly measured the expression of the ER stress-related proteins including Bip, p-PERK, p-eIF2α, ATF4, CHOP, IRE1α, TRAF2 and p-JNK through western blot assay. After treatment with DMAS at the indicated concentrations for 24 hours, expression levels of Bip, p-PERK, p-eIF2α, ATF4, CHOP, IRE1α and p-JNK were upregulated in a dose-dependent method, whereas the expression of TRAF2 was downregulated in a concentration-dependent method (Figure 4). These results indicated that DMAS activated ER stress in human lung adenocarcinoma cells.

To further clarify whether DMAS-induced autophagy depends on ER stress-mediated PERK-eIF2α-ATF4-CHOP and IRE1-TRAF2-JNK signaling pathways, we transfected PC-9 and A549 cells with CHOP siRNA to inhibit CHOP expression. We detected CHOP and LC3B-II levels after treatment with DMAS (15 μmol/L) for 24 hours by western blot. The results showed that the level of LC3B-II significantly decreased after DMAS exposure in CHOP-knockdown cells compared with control-knockdown cells (Figure 5A). Then, we silenced ATF4 expression by siRNA and evaluated the levels of CHOP and LC3B-II expression. After incubation with DMAS (15 μmol/L) for 24 hours, the expression levels of CHOP and LC3B-II also dramatically decreased in ATF4-knockdown cells compared with control-knockdown cells (Figure 5B). Furthermore, we pretreated PC-9 and A549 cells with specific inhibitor for JNK (SP600125) for 1 hour followed by incubation with DMAS (15 μmol/L) for another 24 hours and then detected the expressions of p-JNK and LC3B-II. The results showed that coincubation with DMAS and SP600125 significantly decreased

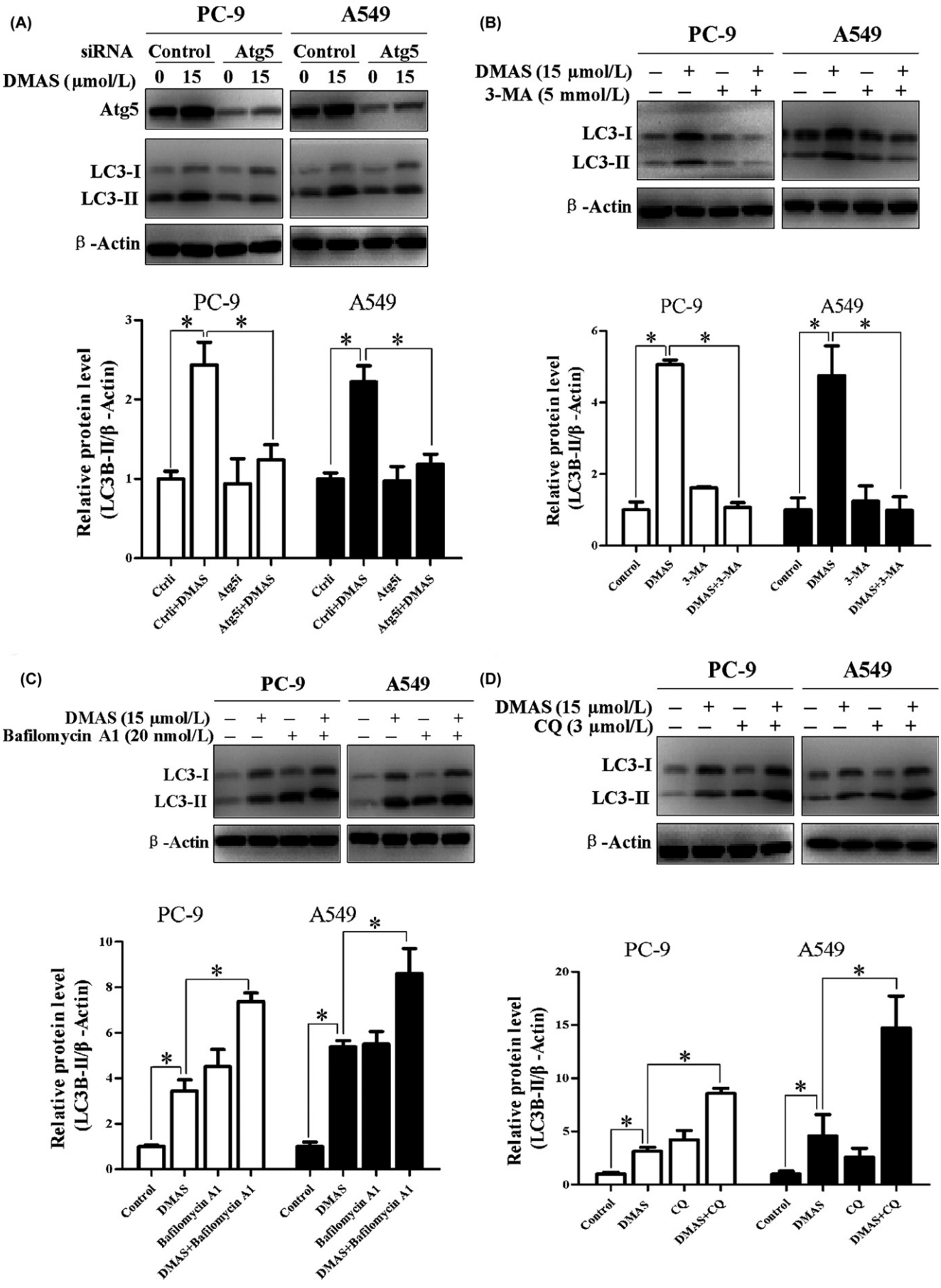

(A)
PC-9
A549
siRNA
Control
Atg5
DMAS (μmol/L)
0 15 0 15 0 15 0 15
Atg5
LC3-I
LC3-II
β -Actin
Relative protein level (LC3B-II/β -Actin)
Ctrli
Ctrli+DMAS
Atg5i
Atg5i+DMAS
(B)
DMAS (15 μmol/L)
3-MA (5 mmol/L)
Control
DMAS
3-MA
DMAS+3-MA
(C)
Bafilomycin A1 (20 nmol/L)
Bafilomycin A1
DMAS+Bafilomycin A1
(D)
CQ (3 μmol/L)
CQ
DMAS+CQ

FIGURE 3 β,β-Dimethylacrylshikonin (DMAS) induces autophagy flux in human lung adenocarcinoma cells. A, PC-9 and A549 cells were transfected with control siRNA or Atg5 siRNA and subsequently exposed to DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were examined by western blot assay. B, PC-9 and A549 cells were pretreated with 3-methyladenine (3-MA; 5 mmol/L) for 1 h, and then coincubated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were detected by western blot assay. C, PC-9 and A549 cells were pretreated with Bafilomycin A1 (20 nmol/L) for 1 h, and then cotreated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were analyzed by western blot assay. D, PC-9 and A549 cells were pretreated with chloroquine (CQ; 3 μmol/L) for 1 h, and then coincubated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were studied by western blot assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group

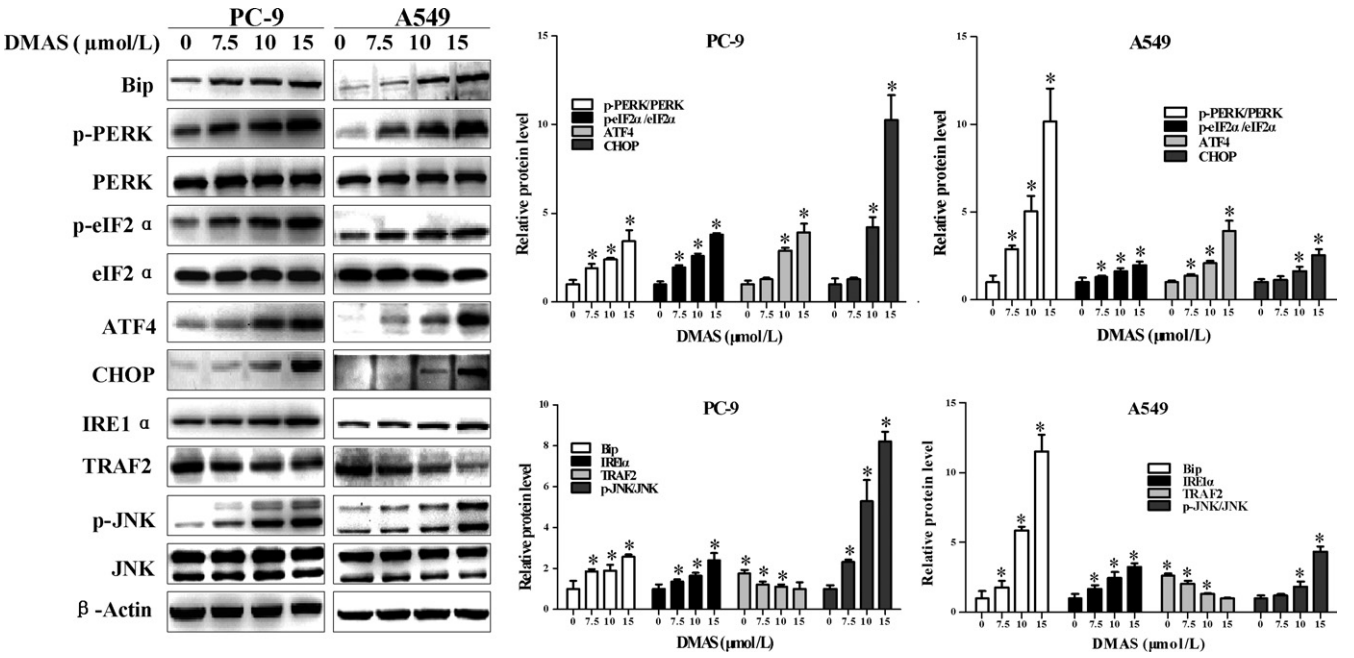
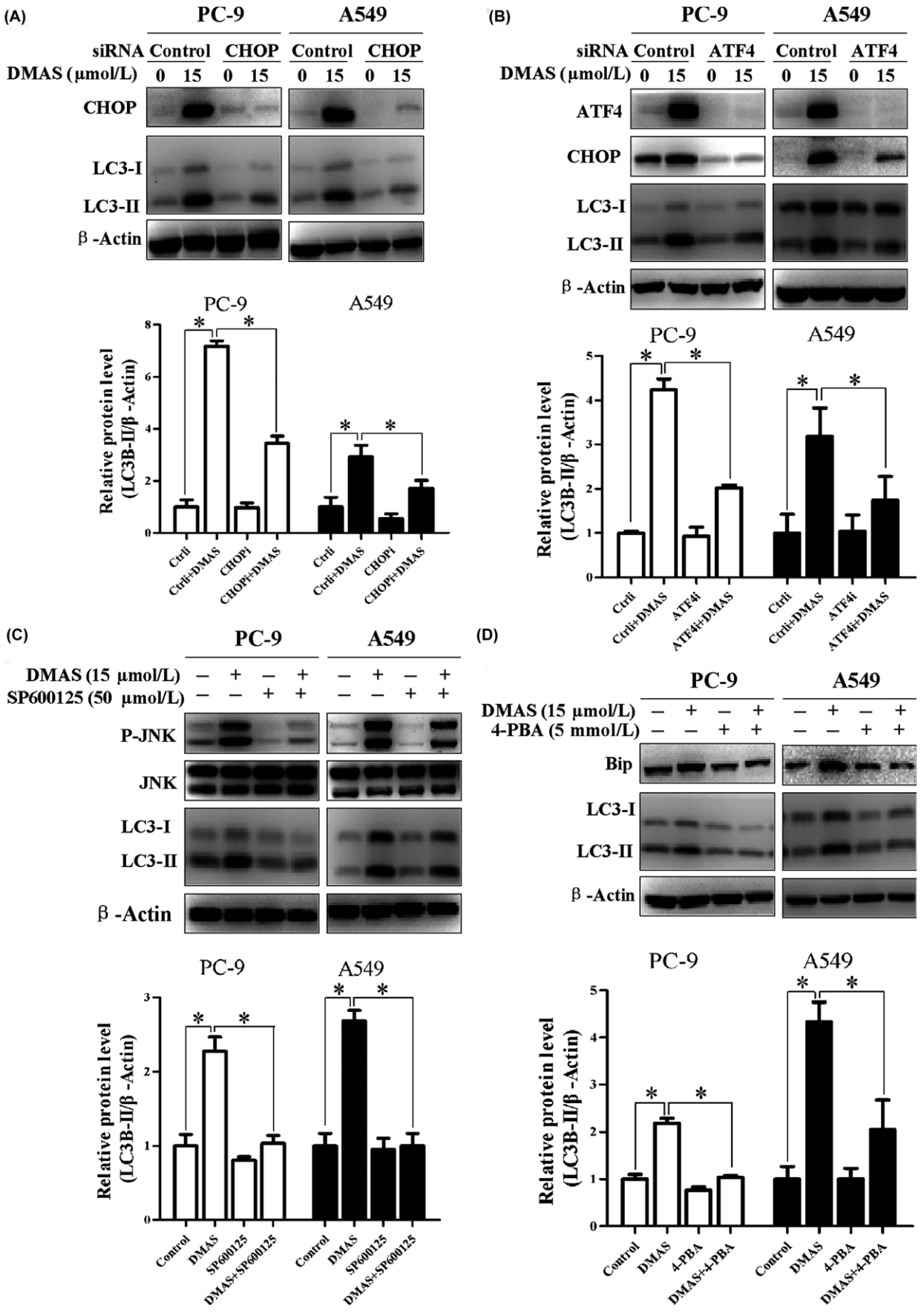


FIGURE 4 β,β-Dimethylacrylshikonin (DMAS) induces endoplasmic reticulum (ER) stress in human lung adenocarcinoma cells. PC-9 and A549 cells were incubated with DMAS for 24 h. Expression levels of indicated proteins were evaluated by western blot assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group

the expressions of p-JNK and LC3B-II in comparison to treatment with DMAS alone (Figure 5C). In addition, we cotreated PC-9 and A549 cells with DMAS (15 μmol/L) and ER stress antagonist 4-PBA (5 mmol/L) for 24 hours and then measured the expressions of Bip and LC3B-II. This showed that combination treatment using DMAS and 4-PBA downregulated the levels of Bip and LC3B-II more than that of DMAS treatment alone (Figure 5D). Thus, DMAS induced autophagy through the ATF4-CHOP and IRE1-TRAF2-JNK signaling pathways. In summary, these data showed that DMAS induced autophagy in human lung adenocarcinoma cells by stimulating the ER stress signaling pathway.

3.4 | Autophagy plays a protective role in DMAS-treated human lung adenocarcinoma cells

As autophagy plays a paradoxical role in cancer cell survival and cell death,[23] we next investigated the role of autophagy in DMAS-induced cell death in human lung adenocarcinoma cells. To examine whether DMAS-induced autophagy promotes or attenuates apoptosis in human lung adenocarcinoma cells, we transfected PC-9 and A549 cells with Atg5 siRNA to block the expression of Atg5. We then measured the cleavage of caspase-3 and PARP after treatment with DMAS (15 μmol/L) for 24 hours. Cleavage of caspase-3 and PARP in Atg5-depleted cells was enhanced after treatment with DMAS compared with the control cells (Figure 6A). Moreover, DMAS treatment induced 31.1% apoptosis in Atg5-knockdown cells, whereas it caused only 23.6% apoptosis in control-knockdown cells when evaluated by Annexin V-FITC/PI double staining and flow cytometry analysis (Figure 6B). Next, we assessed the effects of combination treatment with DMAS and autophagy inhibitors on cell apoptosis in PC-9 and A549 cells. The results showed that treating PC-9 and A549 cells with DMAS and autophagy inhibitors (3-MA, CQ or bafilomycin A1) increased the expression of cleaved caspase-3 and cleaved PARP more than treating PC-9 and A549 cells with DMAS alone (Figure 6C,E,G). Additionally, Annexin V-FITC/PI double-stained assays showed that coincubation with DMAS and autophagy inhibitors (3-MA, CQ or bafilomycin A1) resulted in a significantly greater number of apoptotic cells than treatment with DMAS alone (Figure 6D,F,H). Because DMAS induces autophagy through the ER stress-related ATF4-CHOP and IRE1-TRAF2-JNK axes, we also observed cell


(A)
PC-9
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DMAS (μmol/L) 0 15 0 15 0 15 0 15
CHOP
LC3-I
LC3-II
β -Actin
PC-9
A549
Relative protein level (LC3B-II/β -Actin)
Ctrli
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(B)
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DMAS (μmol/L) 0 15 0 15 0 15 0 15
ATF4
CHOP
LC3-I
LC3-II
β -Actin
PC-9
A549
Relative protein level (LC3B-II/β -Actin)
Ctrli
Ctrli+DMAS
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PC-9
A549
DMAS (15 μmol/L) – + – + – + – +
SP600125 (50 μmol/L) – – + + – – + +
P-JNK
JNK
LC3-I
LC3-II
β -Actin
PC-9
A549
Relative protein level (LC3B-II/β -Actin)
Control
DMAS
SP600125
DMAS+SP600125
(D)
PC-9
A549
DMAS (15 μmol/L) – + – + – + – +
4-PBA (5 mmol/L) – – + + – – + +
Bip
LC3-I
LC3-II
β -Actin
PC-9
A549
Relative protein level (LC3B-II/β -Actin)
Control
DMAS
4-PBA
DMAS+4-PBA

FIGURE 5 β,β-Dimethylacrylshikonin (DMAS) induces autophagy by endoplasmic reticulum (ER) stress-evoked PERK-eIF2α-ATF4-CHOP and IRE1-TRAF2-JNK upregulation. A, PC-9 and A549 cells were transfected with control siRNA or CHOP siRNA for 48 h, and then exposed to DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were detected by western blot assay. B, PC-9 and A549 cells were transfected with control siRNA or ATF4 siRNA for 48 h, and then incubated with DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were analyzed by western blot assay. C, PC-9 and A549 cells were pretreated with SP600125 (50 μmol/L) for 1 h, and then cotreated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were examined by western blot assay. D, PC-9 and A549 cells were pretreated with 4-phenylbutyrate (4-PBA; 5 mmol/L) for 30 min, and then cotreated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were studied by western blot assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group

apoptosis in CHOP- or ATF4-depleted cells treated with DMAS. We knocked down CHOP or ATF4 expression by siRNA and then detected the cleavage of caspase-3 and PARP after treatment with DMAS (15 μmol/L) in PC-9 and A549 cells for 24 hours. Expression levels of cleaved caspase-3 and cleaved PARP in CHOP- or ATF4-knockdown cells were augmented after treatment with DMAS compared with the control cells (Figure 7A,B). Furthermore, we evaluated the roles of combination treatment with DMAS and SP600125 (specific inhibitor for JNK) or 4-PBA (ER stress antagonist) on cell apoptosis in PC-9 and A549 cells. As shown in Figure 7C,D, combination treatment using DMAS and SP600125 or 4-PBA significantly upregulated the expression levels of cleaved caspase-3 and cleaved PARP than that of DMAS treatment alone. However, the DMAS-induced cleavage of caspase-3 and PARP was not influenced by prior treatment with SP600125 in A549 cells (Figure 7C). Collectively, these data confirmed that autophagy contributed to protecting cells from DMAS-induced apoptosis in human lung adenocarcinoma cells.

4 | DISCUSSION

β,β-Dimethylacrylshikonin is a plant-derived natural product and shows strong anti-tumor effects.[6] We previously reported that DMAS inhibited growth and induced apoptosis in human gastric cancer SGC-7901 cells.[9] In the present study, we reported for the first time the multifaceted antitumor activities of DMAS on human lung adenocarcinoma cells. DMAS substantially decreased the viability of PC-9 cells in both dose-dependent and time-dependent methods. Apoptosis, autophagy, as well as ER stress, were induced by DMAS treatment.

The induction of apoptosis is thought to be a key mechanism for most anti-tumor therapies. Caspases, a family of proteases, are responsible for the execution of apoptosis.[24] Flow cytometry with Annexin V-FITC/PI double staining suggested that DMAS induced apoptosis in PC-9 cells, which was supported by the results of DAPI staining assay and increased amount of cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, and cleaved PARP. Furthermore, DMAS-induced cleavage of caspase-3 and PARP in PC-9 cells was almost completely blocked by pretreatment with the pan-caspase inhibitor Z-VAD-FMK. These data suggested that DMAS induced apoptosis of PC-9 cells through caspase activation. Consistently, our previous study also showed that DMAS induced apoptosis in human lung adenocarcinoma A549 cells by the caspase-dependent apoptotic pathway.[15]

Autophagy is an evolutionarily conserved cellular self-digesting process in which damaged and harmful cellular components are engulfed into cytosolic double-membrane vesicles termed autophagosomes, and end up in the lysosome.[25] To determine whether DMAS induces autophagy in PC-9 and A549 cells, we used various assay methods. MDC staining and TEM showed the accumulation of autophagic vacuoles. Western blot assay indicated that the level of autophagy marker LC3B-II was increased in a time- and dose-dependent method and the expression of autophagy-related proteins Beclin-1 and Atg5 were also increased in a concentration-dependent way. We also used tandem Stub-RFP-Sens-GFP-LC3 fluorescence assay to confirm the formation of autophagosomes in DMAS-treated PC-9 and A549 cells. In addition, we examined whether DMAS could induce the autophagic flux in PC-9 and A549 cells. Coincubation with DMAS and autophagy inhibitor 3-MA or genetic inhibition of autophagy by knocking down the Atg5 gene decreased DMAS-induced LC3B-II formation in PC-9 and A549 cells. On the contrary, cotreatment with DMAS and autophagy inhibitors, such as bafilomycin A1 or CQ, increased DMAS-induced LC3B-II accumulation in PC-9 and A549 cells. Overall, these findings suggested that DMAS could induce autophagy in human lung adenocarcinoma cells.

Growing evidence indicates that autophagy can be induced by activating ER stress.[19,26,27] ER stress activates the PERK-eIF2α-ATF4-CHOP and IRE1-TRAF2-JNK signaling axes of the UPR, which play a vital role in the regulation of ER stress-induced autophagy.[18] Actually, many anticancer compounds have been reported to function primarily through stimulation of ER stress.[28,29] In our study, we showed that DMAS could upregulate ER stress-associated proteins such as Bip, p-PERK, p-eIF2α, ATF4, CHOP, IRE1α and p-JNK in a dose-dependent way, and downregulate TRAF2 in a concentration-dependent way. Moreover, using siRNA against ATF4 or CHOP and inhibitors of ER stress in combination with DMAS, we confirmed that the PERK-eIF2α-ATF4-CHOP and IRE1-TRAF2-JNK signaling axes of the UPR were the crucial mediator of DMAS-induced autophagy in PC-9 and A549 cells. Collectively, these data showed that DMAS induced autophagy through activation of ER stress in human lung adenocarcinoma cells.

The pro-survival and pro-death functions of autophagy in cancer cells highly depend on cell types and inducers.[30,31] In the present study, we investigated the relationship between autophagy and apoptosis in DMAS-treated PC-9 and A549 cells and

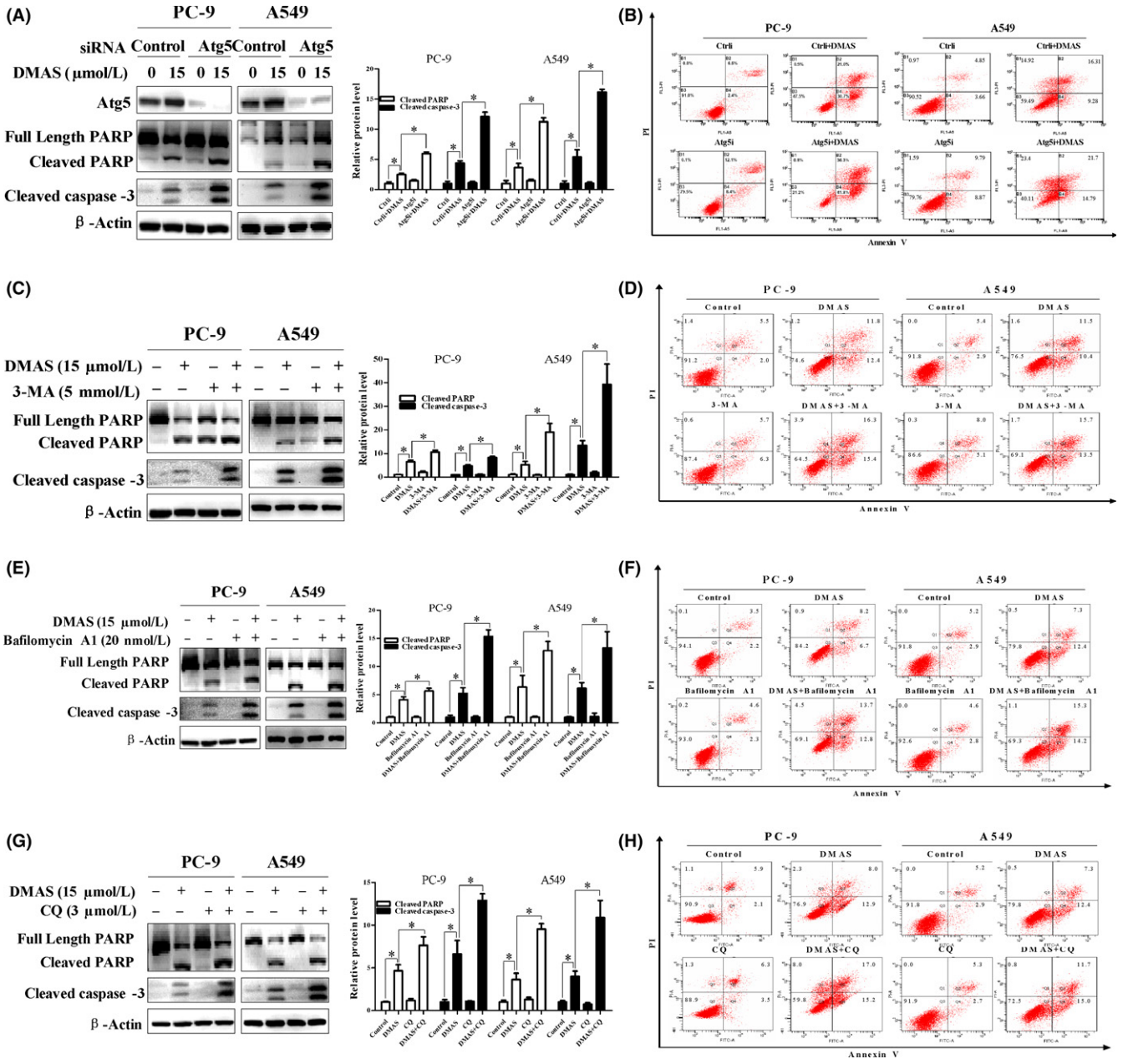
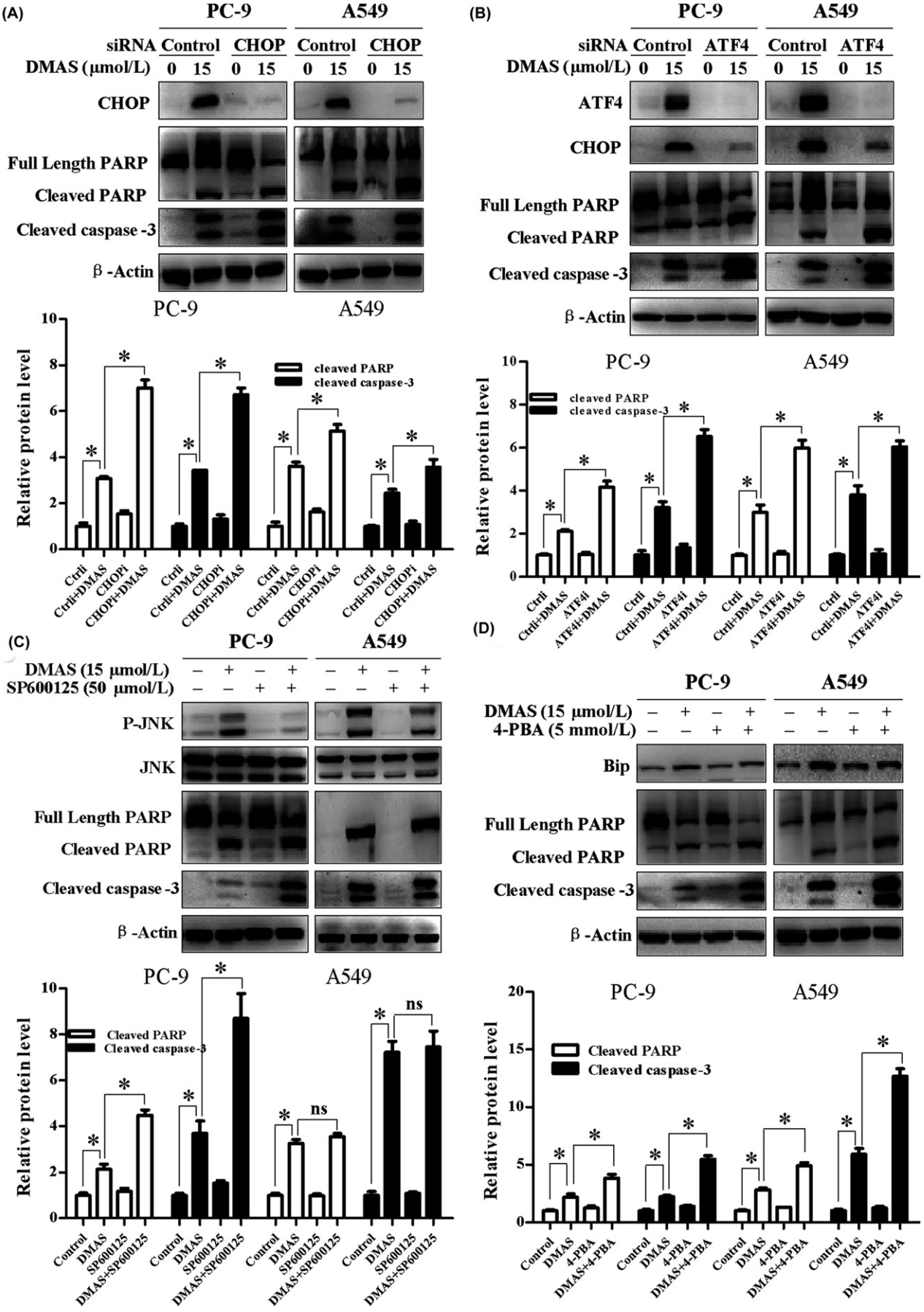


FIGURE 6 Autophagy plays a protective role in β,β-dimethylacrylshikonin (DMAS)-treated human lung adenocarcinoma cells. (A,B) PC-9 and A549 cells were transfected with control siRNA or Atg5 siRNA for 48 h, and then treated with DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were measured by western blot assay. Apoptotic cells were studied through the Annexin V-FITC/propidium iodide (PI) staining assay. (C,D) PC-9 and A549 cells were pretreated with 3-methyladenine 3-MA; 5 mmol/L) for 1 h, and then coincubated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were evaluated by western blot assay. Apoptotic cells were detected through the Annexin V-FITC/PI staining assay. (E,F) PC-9 and A549 cells were pretreated with Bafilomycin A1 (20 nmol/L) for 1 h, and then cotreated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were analyzed by western blot assay. Apoptotic cells were detected by Annexin V-FITC/PI staining assay. (G,H) PC-9 and A549 cells were pretreated with chloroquine (CQ; 3 μmol/L) for 1 h, and then coincubated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were studied by western blot assay. Apoptotic cells were examined by Annexin V-FITC/PI staining assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group. PARP, poly ADP ribose polymerase

found that when combined with DMAS, apoptosis was enhanced upon knockdown of ATG5 by siRNA or in autophagy inhibitor-treated cells, which indicates that autophagy contributes to promoting cell survival in DMAS-treated human lung adenocarcinoma cells.

To sum up, in the present research, we showed that DMAS induced autophagy through the ER stress response, which played a protective role against DMAS-induced apoptosis in human lung adenocarcinoma cells. Previous studies have reported that autophagy suppression may be a therapeutic approach for cancer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PC-9
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DMAS (μmol/L) 0 15 0 15 0 15 0 15
ATF4
CHOP
Full Length PARP
Cleaved PARP
Cleaved caspase -3
β -Actin
PC-9
A549
Relative protein level
cleaved PARP
cleaved caspase-3
Ctrli
Ctrli+DMAS
ATF4i
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PC-9
A549
DMAS (15 μmol/L) – + – + – + – +
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P-JNK
JNK
Full Length PARP
Cleaved PARP
Cleaved caspase -3
β -Actin
PC-9
A549
Relative protein level
Cleaved PARP
Cleaved caspase-3
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Control
DMAS
SP600125
DMAS+SP600125
(D)
PC-9
A549
DMAS (15 μmol/L) – + – + – + – +
4-PBA (5 mmol/L) – – + + – – + +
Bip
Full Length PARP
Cleaved PARP
Cleaved caspase -3
β -Actin
PC-9
A549
Relative protein level
Cleaved PARP
Cleaved caspase-3
Control
DMAS
4-PBA
DMAS+4-PBA

FIGURE 7 Endoplasmic reticulum (ER) stress inhibition confers sensitivity to β,β-dimethylacrylshikonin (DMAS) in human lung adenocarcinoma cells. A, PC-9 and A549 cells were transfected with control siRNA or CHOP siRNA for 48 h, and then incubated with DMAS (15 μmol/L) for another 24 h. Expression levels of indicated proteins were studied by western blot assay. B, PC-9 and A549 cells were transfected with control siRNA or ATF4 siRNA for 48 h, and then exposed to DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were measured by western blot analysis. C, PC-9 and A549 cells were pretreated with SP600125 (50 μmol/L) for 1 h, and then coincubated with DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were examined by western blot assay. D, PC-9 and A549 cells were pretreated with 4-phenylbutyrate (4-PBA; 5 mmol/L) for 30 min, and then cotreated with DMAS (15 μmol/L) for 24 h. Expression levels of indicated proteins were detected by western blot assay. All data are expressed as mean ± SD. *$P < .05$ compared to the control group. ns, no significant difference. PARP, poly ADP ribose polymerase

treatment.[32,33] We predict that combination treatment with DMAS and pharmacological autophagy inhibitors will be a safer and more effective therapeutic strategy for cancer treatment.

ACKNOWLEDGMENTS

This work was financially supported by a grant from the National Natural Science Foundation of China (No. 81403143) and the Excellent Youth Talent Program of Zhejiang Provincial Hospital of Traditional Chinese Medicine (No. 2D01417).

CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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How to cite this article: Wang H, Zhang G. Endoplasmic reticulum stress-mediated autophagy protects against β,β-dimethylacrylshikonin-induced apoptosis in lung adenocarcinoma cells. *Cancer Sci*. 2018;109:1889–1901. https://doi.org/10.1111/cas.13616