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ORIGINAL ARTICLE

Salidroside Induces Apoptosis and Autophagy in Gastric Cancer Cells via Regulation of Mitogen-Activated Protein Kinases Signaling Pathway

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ABSTRACT

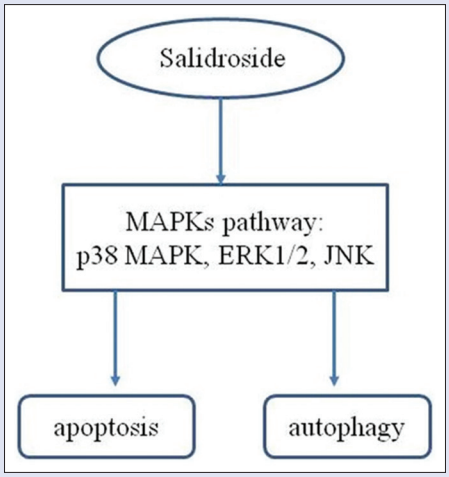
Background: Salidroside, an active ingredient of *Rhodiola rosea*, exhibits antiproliferative effect in gastric cancer (GC) cells. However, the involvement of salidroside in apoptosis and autophagy of GC cells has not been elucidated. **Materials and Methods:** Cell viability of BGC-823 cells was assessed by CCK-8 assay, and apoptosis was analyzed with terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling assay and flow cytometry. The apoptosis- and autophagy-associated proteins and mitogen-activated protein kinases (MAPKs) were determined by Western blot analysis. Autophagy was evaluated by green fluorescent protein-fused LC3 punctate formation. **Results:** Salidroside inhibited proliferation and promoted apoptosis and autophagy of BGC-823 cells in a dose-dependent manner. In addition, salidroside enhanced the phosphorylation of p38 MAPK, ERK1/2, and JNK. Furthermore, inhibition of MAPKs significantly abolished the effects of salidroside in BGC-823 cells. **Conclusion:** Salidroside induced apoptosis and autophagy of BGC-823 cells via activation of MAPK signaling pathway.

Key words: Apoptosis, autophagy, gastric cancer, mitogen-activated protein kinase, salidroside

SUMMARY

- Salidroside promotes GC cell apoptosis and autophagy in a dose-dependent manner
- Salidroside increases the phosphorylation of p38 mitogen-activated protein kinase, ERK1/2, and JNK
- Inhibition of mitogen-activated protein kinases abolishes salidroside-induced cell apoptosis and autophagy in GC cells.

Abbreviations used: FITC: Fluorescein isothiocyanate; GC: Gastric cancer; JAK2: Janus kinase 2; MAPKs: Mitogen-activated protein kinases; PI: Propidine iodide; SD: Standard deviation; STAT3: Signal transducer and activator of transcription 3; TUNEL: Terminal transferees dUTP nick-end labeling.



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INTRODUCTION

In China, gastric cancer (GC) is one of the most common malignant tumors and a major cause for cancer-related deaths.[1] GC is difficult to be detected at an early stage because of its inconspicuous clinical manifestations. Radical surgery is one of the curative treatments for GC as it is characterized by rapid development, short survival period, and poor prognosis.[2] However, relapse represents the major cause of treatment failure. Radiotherapy and chemotherapy can be used as adjuvant therapies before or after surgery, but they cause serious side effects.[3] At present, despite some of the promising developments in the treatment of GC, the overall outcome is still not ideal, and the overall survival has hardly been improved.[4] Therefore, it is necessary to develop more effective treatments for GC. At present, traditional Chinese medicine is used frequently in the comprehensive treatment of GC.

Salidroside is the primary active ingredient extracted from *Rhodiola rosea*.[5] Several lines of evidence suggest that salidroside shows antitumor

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activities via induction of apoptosis and autophagy. Fan *et al.* reported anticarcinogenic activities of salidroside by inducing a pro-death autophagy and apoptosis in human colorectal cancer cells.[6] Zeng *et al.* found that salidroside is a potent inducer of apoptosis and autophagy in human chondrosarcoma through TFEB-dependent autophagy.[7] Li *et al.* showed that salidroside induced the apoptosis and autophagy of bladder cancer cells via PI3K/Akt signaling pathway.[8] Furthermore, salidroside inhibited the malignant behaviors of GC cells.[9] However, the involvement of salidroside in apoptosis and autophagy of GC cells has not been elucidated. Therefore, in this study, we aimed to investigate the effects of salidroside-induced autophagy in GC cells and its underlying mechanisms.

MATERIALS AND METHODS

Cell culture and treatment

Human GC cell line BGC-823, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), was cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100 mg/mL streptomycin (Gibco, Carlsbad, CA, USA) at 37°C with 5% CO_2. Cells were preconditioned with inhibitor of autophagy 3-methyladenine (3-MA; 10 mM; No. M9281) for 30 min or the mitogen-activated protein kinase (MAPK) p38 inhibitor SB203580 (No. S8307; 10 mM), the ERK1/2 inhibitor U0126 (No. U120; 10 mM), and the JNK inhibitor SP600125 (No. S5567; 10 mM) (Sigma-Aldrich, Shanghai, China) for 48 h before treatment with salidroside (No.

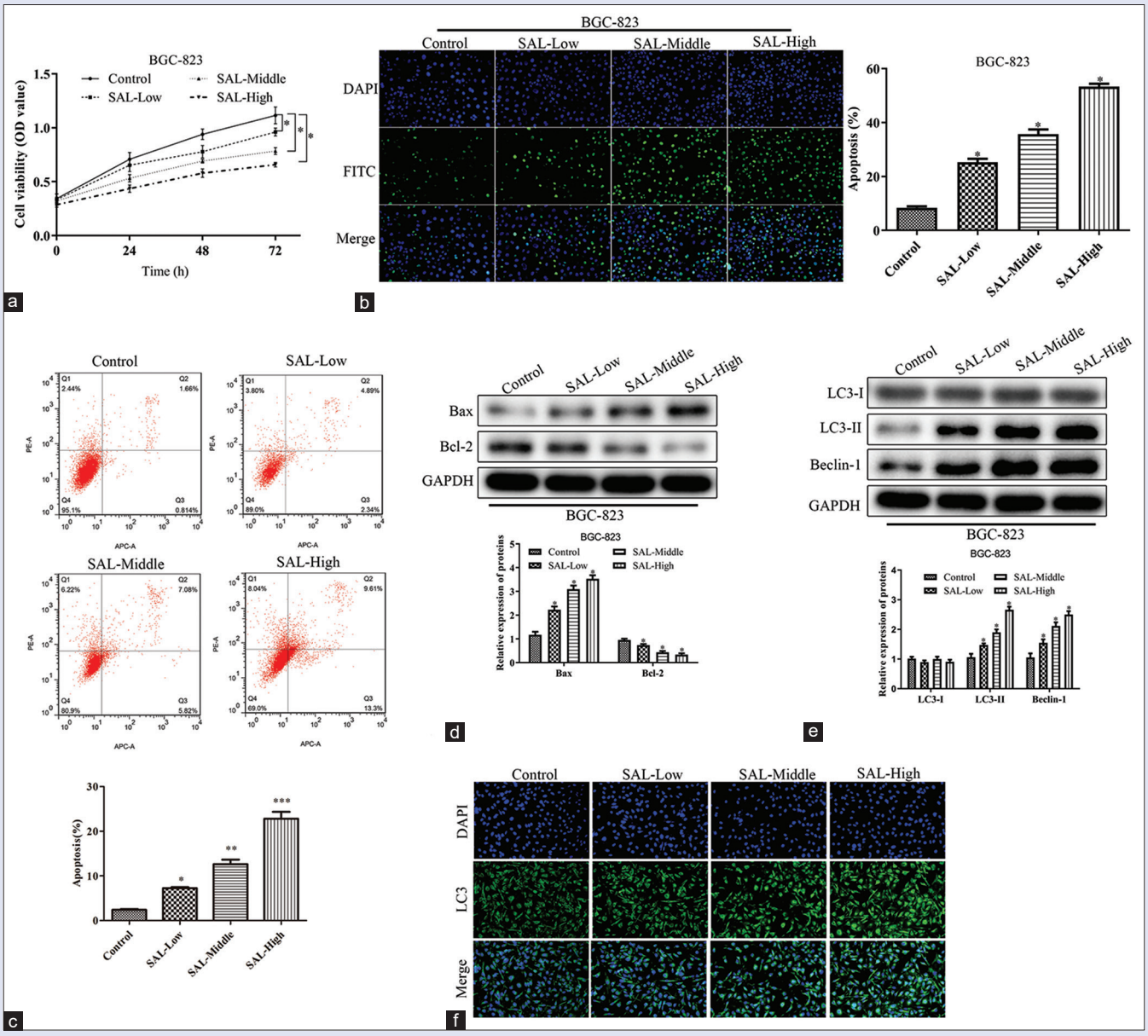

Figure 1: Effects of salidroside on apoptosis, autophagy and mitogen-activated protein kinases pathway in GC cells. The CCK-8 viability assay (a), Terminal transferees dUTP nick end labeling apoptosis detection (b), apoptotic rate using a FITC Annexin V apoptosis detection kit using flow cytometry (c), the expressions of Bax, Bcl-2, p-p38, p38, p-ERK1/2, ERK1/2, p-JNK, JNK, LC3-I, LC3-II, and Beclin-1 (d and e) and the formation of autophagic vacuoles as determined by immunofluorescent staining for LC3 (f) in salidroside-treated BGC-823 cells. Statistically significant differences were indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

43,866; analytical grade; purity ≥98.0%). Salidroside was diluted in cell culture medium to obtain its final concentration of 0.5, 1, and 2 mM and cultured for the indicated time periods. Vehicle control was maintained by cells that were treated with a fresh medium with 0.1% dimethylsulfoxide vehicle.

CCK-8 cell viability assay

Cell viability was determined by CCK-8 assay

Briefly, BGC-823 cells were seeded in 96-well plates at a density of 1×10^4 cells/mL. Then, salidroside was added at different concentrations (0.5, 1, and 2 mM). After washing thrice with phosphate-buffered saline (PBS), cells were incubated with CCK-8 (Beyotime, Shanghai, China) solution (10 μL per well) at 37°C for 4 h. Then, the absorbance was measured at 450 nm by a microplate reader. The cell viability was expressed as a percentage of the controls.

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling staining

After treatment with different concentrations of salidroside (0.5, 1, and 2 mM), BGC-823 cells were fixed in 4% paraformaldehyde and blocked with 0.2% Triton-X 100. The Terminal transferees dUTP nick end labeling (TUNEL) assay kit (Roche Diagnostics, Mannheim, Germany) was prepared immediately before use according to the manufacturer's protocol. Subsequent to washing with PBS, the nuclei were counterstained with DAPI. The TUNEL-positive apoptotic cells were counted by a fluorescence microscope at ×400 magnification.

Flow cytometry

Cell apoptosis was also analyzed by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences; San Jose, CA, USA) according to the manufacturer's protocols. Briefly, after incubating with different concentrations of salidroside (0.5, 1, and 2 mM), BGC-823 cells were collected, washed with cold PBS, and stained with the binding buffer containing Annexin V-FITC and propidine iodide (PI) at 4°C under darkness for 15 min. Finally, cells were recorded using flow cytometry (Beckman Coulter, Fullerton, CA, USA).

Immunofluorescence

BGC-823 cells (8×10^4 cells/mL) were seeded in a 35-mm diameter Petri dish covered with a glass slide (microscope cover glass, 18 mm × 18 mm) and maintained at 37°C with 5% CO_2 for 24 h. These cells were transiently transfected with a green fluorescent protein (GFP)-fused LC3 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specification. After 48 h of posttransfection, the slides were rinsed thrice with PBS, fixed in 4% paraformaldehyde for 10 min, again washed thrice with PBS, and sealed with anti-fluorescence quenching mounting medium (15 μL). Fluorescence levels of GFP-LC3 were detected by using laser confocal microscopy (CLSM; Leica, Wetzlar, Germany; magnification 60×). To quantify autophagic cells, we counted the number of autophagic cells demonstrating GFP-LC3 dots (≥10 dots/cell) among 200 GFP-positive cells.

Western blot

Total protein was extracted from the homogenate of BGC-823 cells. The lysate was centrifuged to collect supernatant, and the protein concentration was determined by bicinchoninic acid assay. Total protein (50 μg) was separated by SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat milk for 1 h and incubated with primary antibodies against Bax (#5023), Bcl-2 (#4223), p-p38 (#4511), p38 (#8690), p-ERK1/2 (#4370), ERK1/2 (#4695),

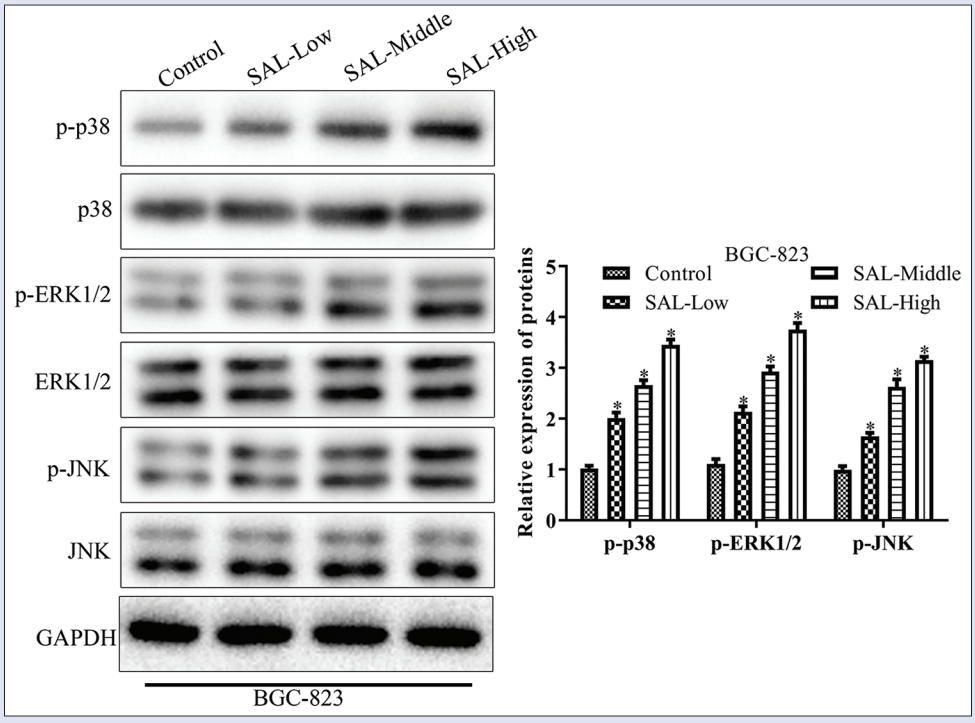

Figure 2: Effects of salidroside on mitogen-activated protein kinases pathway in GC cells. The protein expression of p-ERK1/2, ERK1/2, p-JNK, and JNK in salidroside-treated BGC-823 cells. Statistically significant differences were indicated (*$P < 0.05$)

p-JNK (#4668), JNK (#9252), LC3-I (#4599), LC3-II (#3868), Beclin-1 (#3495), and GAPDH (#5174) (1:1000 dilution) overnight at 4°C, followed by 2-h incubation with FITC-labeled IgG secondary antibody (#14708), which were obtained from Cell Signaling Technology (Boston, MA, USA). The bands were observed by a chemiluminescent detection system. For each sample, band intensities were normalized to GAPDH.

Statistical analyses

Data were expressed as mean ± standard deviation of three independent experiments. Comparisons were assessed by the analysis of variance using SPSS 19.0 (IBM, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Effects of salidroside on apoptosis and autophagy in gastric cancer cells

To examine the potential antitumor activity of salidroside against GC, we incubated BGC-823 cells with different concentrations (0.5, 1, and 2 mM) of salidroside. Cell viability was measured by CCK-8 assay. As shown in Figure 1a, salidroside inhibited cell viability in a time- and dose-dependent manner with an IC_{50} value of 1.87 mM. Salidroside induced apoptosis in a dose-dependent manner as demonstrated by TUNEL assay [Figure 1b] and flow cytometry [Figure 1c]. Furthermore, salidroside upregulated the expression of Bax and downregulated the expression of Bcl-2 [Figure 1d]. In addition, autophagy-related molecules such as LC3-II and Beclin-1 were markedly upregulated by salidroside in a dose-dependent manner [Figure 1e]. Salidroside induces the formation of autophagic vacuoles as determined by immunofluorescent staining for LC3 [Figure 1f]. Collectively, these results show that salidroside increased apoptosis and autophagy in BGC-823 cells.

Effects of salidroside on mitogen-activated protein kinases pathway in gastric cancer cells

In this study, in addition to the underlying mechanism, we investigated whether salidroside can induce apoptosis and autophagy in BGC-823 cells. The MAPK signaling pathway is a key pathway related to apoptosis and autophagy;[10,11] therefore, we investigated whether this pathway plays a central role in salidroside-mediated apoptosis and autophagy. As shown in Figure 2, increasingly higher concentrations of salidroside significantly stimulated the phosphorylation of p38, ERK1/2, and JNK; however, there was no change observed in the total quantity of p38, ERK1/2, and JNK proteins. In summary, it was clearly suggested that

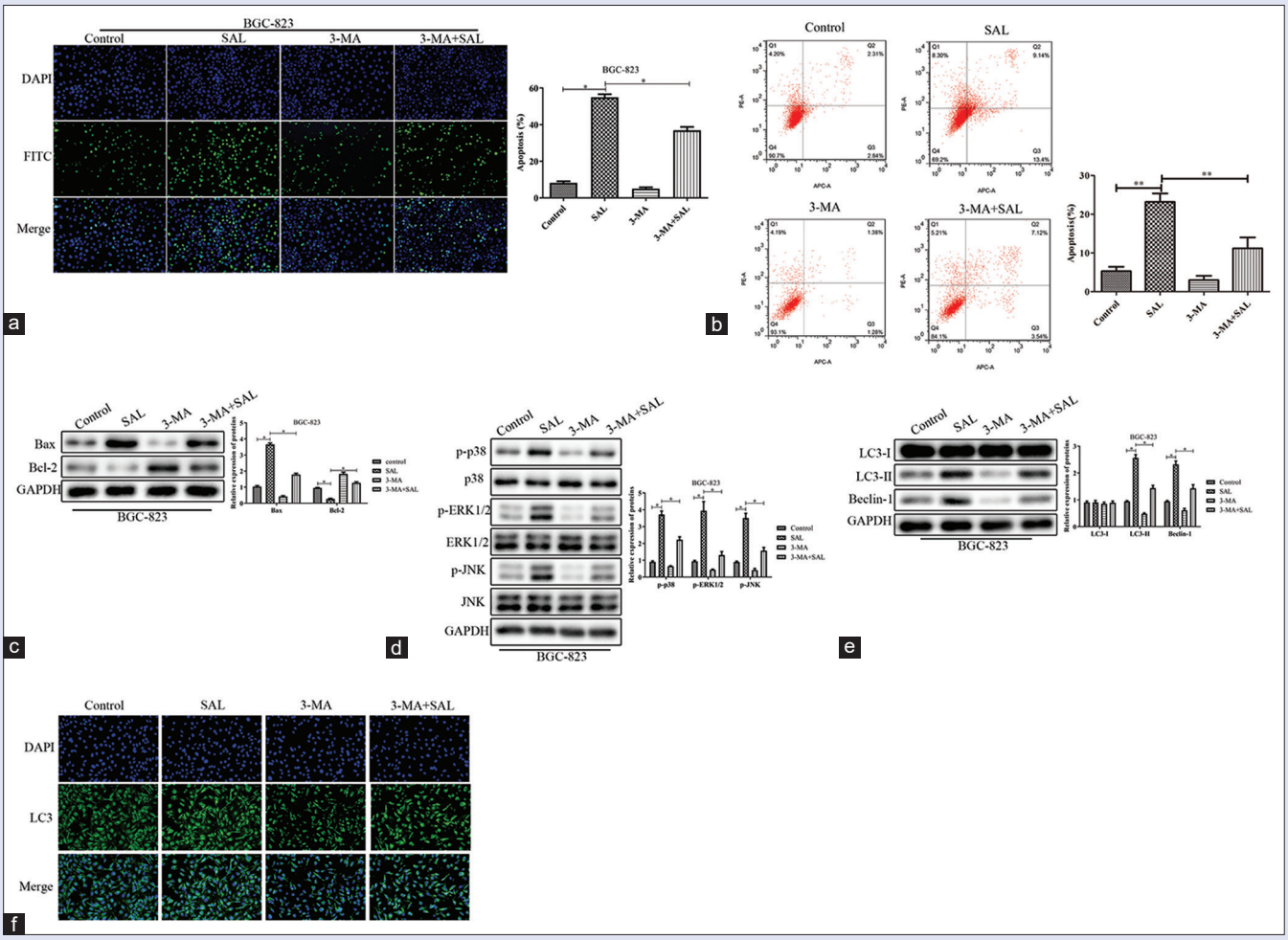

Figure 3: Effects of autophagy inhibition on salidroside-induced apoptosis and autophagy in GC cells. Apoptosis detection (a and b) and the protein levels of Bax, Bcl-2, p-p38, p38, p-ERK1/2, ERK1/2, p-JNK, JNK, LC3-I, LC3-II, and Beclin-1 (c-e) and LC3 immunofluorescent staining (f) in BGC-823 cells treated with 10 mM 3-MA for 30 min alone or in combination with salidroside. Statistically significant differences were indicated (*$P < 0.05$, **$P < 0.01$)

salidroside induced apoptosis and autophagy in BGC-823 cells via activation of the MAPK signaling pathway.

Effects of autophagy inhibition on salidroside-induced apoptosis in gastric cancer cells

As mentioned above, we found that salidroside increased apoptosis and autophagy in BGC-823 cells. Then, we used 3-MA, an inhibitor of autophagy to determine the relationship between apoptosis and autophagy after treating BGC-823 cells with salidroside. Our results showed that cell apoptosis was inhibited by 3-MA as evidenced by reduced number of TUNEL-positive apoptotic cells [Figure 3a], reduced apoptotic rate [Figure 3b], downregulated levels of Bax protein, and upregulated levels of Bcl-2 [Figure 3c]. Meanwhile, 3-MA treatment inhibited the phosphorylated levels of p38 MAPK, ERK1/2, and JNK without a change in their total protein levels [Figure 3d]. Furthermore, the levels of LC3-II/I ratio and Beclin-1 were suppressed by 3-MA [Figure 3e]. However, these effects of 3-MA were abrogated by salidroside co-treatment. Immunofluorescent staining was used to determine the LC3 $^{+}$ autophagic vacuole formation and the results showed that 3-MA prevented salidroside-induced autophagic vacuole formation [Figure 3f]. These data indicate that suppression of autophagy blocks the salidroside-induced apoptosis in BGC-823 cells.

Effects of inhibition of mitogen-activated protein kinases on salidroside-induced apoptosis and autophagy in gastric cancer cells

Next, we investigated whether salidroside-induced autophagy depended on the activation of MAPK pathway. To achieve this, BGC-823

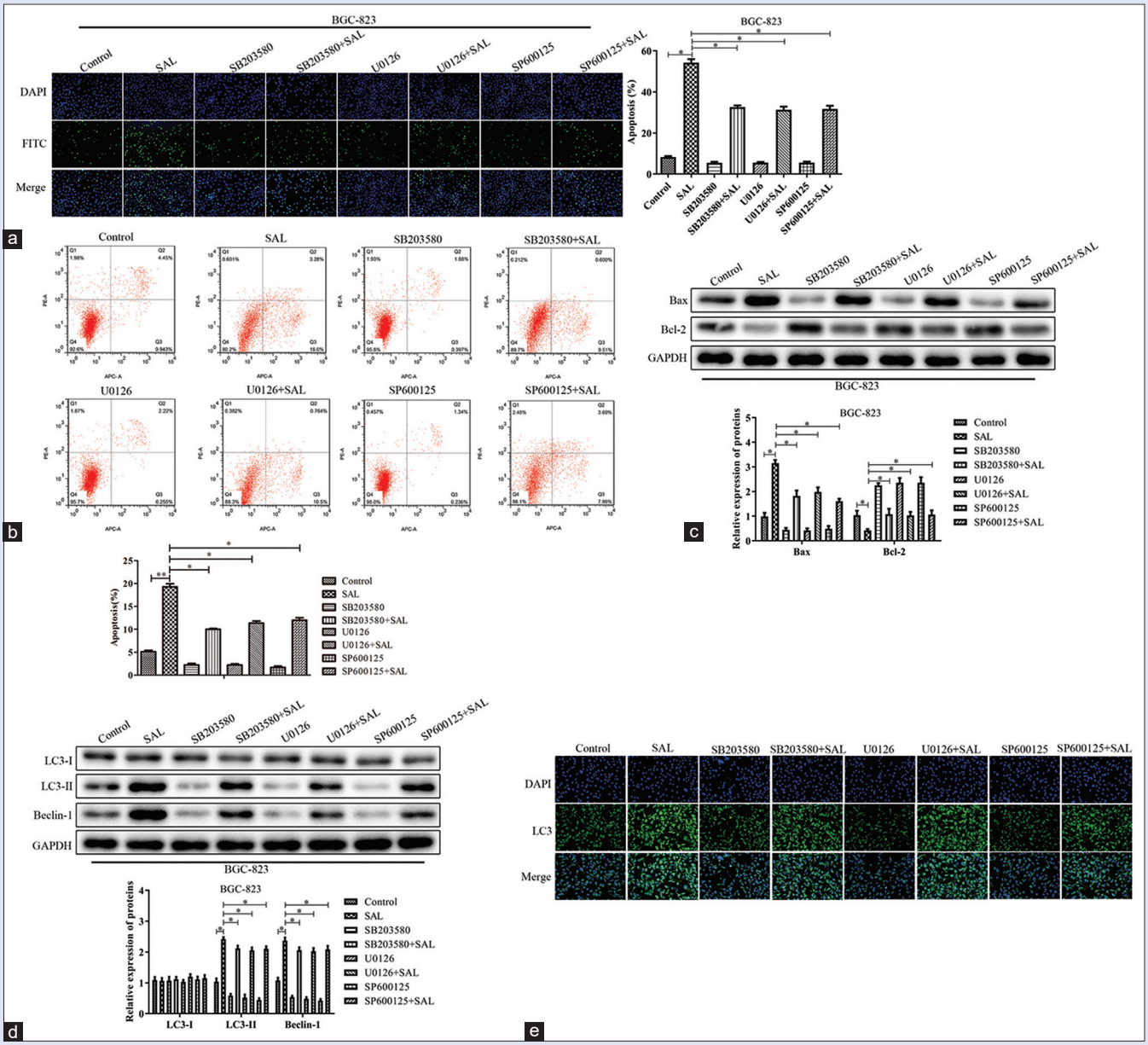

Figure 4: Effects of inhibition of mitogen-activated protein kinases on salidroside-induced apoptosis and autophagy in GC cells. Apoptosis detection (a and b) and the protein levels of Bax, Bcl-2, LC3-I, LC3-II, and Beclin-1 (c and d) and LC3 immunofluorescent staining (e) in BGC-823 cells treated with 10 mM SB203580, U0126, and SP600125 for 48 h alone or in combination with salidroside. Statistically significant differences were indicated (*P < 0.05, **P < 0.01)

cells were treated with SB203580 (an inhibitor of p38), U0126 (an inhibitor of ERK1/2), and SP600125 (an inhibitor of JNK) for 48 h followed by treatment with or without salidroside. As demonstrated by Figure 4a-c, SB203580, U0126, or SP600125 significantly attenuated salidroside-induced cell apoptosis. In addition, these MAPK inhibitors resulted in reduced conversion of LC3-I into LC3-II and decreased the levels of Beclin-1, which were all reversed by salidroside [Figure 4d]. Furthermore, salidroside-induced autophagic vacuole formation was also attenuated by these MAPK inhibitors [Figure 4e]. In summary, these results demonstrated that salidroside induced autophagy in BGC-823 cells via activation of p38 MAPK, ERK1/2, and JNK.

DISCUSSION

In this study, we investigated the antitumor activity of salidroside. Salidroside induced apoptosis in BGC-823 cells via upregulation of autophagy mediated by p38 MAPK, ERK1/2, and JNK signaling pathways.

To the best of our knowledge, salidroside shows potent antitumor activity against various cancers, which may be a promising drug candidate for cancer therapy. For instance, salidroside reduced cell proliferation and induced G1 phase cell cycle arrest and apoptosis of renal cell carcinoma cells in a dose-dependent manner.[12] In human ovarian cancer cells, SKOV3 and A2780 cells, salidroside exerted antiproliferative effects by inducing apoptosis via activation of p53 signaling pathway.[13] Salidroside suppressed migration and invasion and induced apoptosis of poorly differentiated thyroid cancer cells via the inhibition of Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 pathway.[14] However, so far, there is no report about the detailed action of salidroside in GC. Herein, we found that salidroside dose and time dependently inhibited cell proliferation of human GC cells, which is consistent with the previous study.[9] In addition, salidroside induced apoptosis in BGC-823 cells in a dose-dependent manner. However, the pro-apoptotic mechanisms of salidroside in BGC-823 cells have not yet been reported.

Autophagy, also called type II programmed cell death, is a "self-feeding" process involving the degradation of damaged organelles and misfolded proteins in eukaryotes, which plays an important role in physiological and pathological processes in several malignant tumors.[15-17] Autophagy can serve as a mechanism of self-defense by contributing to resistance to therapy.[18,19] In addition to its role in normal physiology, autophagy also plays a key role in pathological processes such as cancer. One of the emerging themes is that in certain types of cancer, autophagy is important to inhibit tumor growth; therefore, the indication of autophagy as a therapeutic approach is actively being tested in clinical trials.[20] The antitumor mechanism of salidroside has been recently reported to involve the induction of autophagy.[6-8] However, the effect of salidroside in GC is not fully understood. Herein, salidroside showed induction of autophagy in BGC-823 cells via upregulation of LC3-II and Beclin-1 in a dose-dependent manner. Conversely, pretreatment of BGC-823 cells with an autophagy inhibitor 3-MA abolished the increase of apoptosis and autophagy induced by salidroside. Collectively, these results indicate that inhibition of autophagy provide a protective mechanism against salidroside-induced apoptosis in GC cells. Our speculation is supported by another report, in which salidroside increased autophagy and apoptosis in human colorectal cancer cells.[6]

MAPKs are intracellular signaling pathways comprised three major members: p38 MAPK, ERK1/2, and JNK. These pathways regulate cell survival, protein synthesis, motility, and so on.[10,21] In recent years, MAPKs have attracted much attention as a promising target for cancer therapy.[22,23] More importantly, several researchers have proposed that the activation of MAPKs is associated with apoptosis and autophagy.[10,11] Our results in this study demonstrated that salidroside treatment elevated the phosphorylation of p38 MAPK, ERK1/2, and JNK in BGC-823 cells in a dose-dependent manner. In contrast, the addition of 3-MA effectively suppressed the activation of p38 MAPK, ERK1/2, and JNK induced by the salidroside. Moreover, pretreatment with p38 MAPK-specific inhibitor (SB203580), ERK1/2-specific inhibitor (U0126), or JNK-specific inhibitor (SP600125) could abrogate salidroside-induced apoptosis and autophagy. Thus, the inhibition of the MAPK signaling pathway contributes, at least in part, to the apoptosis- and autophagy-inducing effect of salidroside in BGC-823 cells.

CONCLUSION

Despite the lack of clinical data and *in vivo* experiments, our finding confirmed the underlying mechanism of salidroside in GC cells via the MAPK signaling pathway. Further investigations are needed to verify the therapeutic action of salidroside in GC development.

Acknowledgements

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Author's contributions

Zhijun Fang and Jiege Huo conceived and designed the experiments; Lingchang Li, FengXia Qin and Guoli Wei performed the experiments; Xiaofei Huang and Rong Ding analyzed the data; Canhong Hu ang Shao Xiang wrote the paper.

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Conflicts of interest

There are no conflicts of interest.

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