Alantolactone enhances gemcitabine sensitivity of lung cancer cells through the reactive oxygen species-mediated endoplasmic reticulum stress and Akt/GSK3β pathway

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Abstract. Lung cancer is one of the leading causes of cancer-associated mortality in China and globally. Gemcitabine (GEM), as a first-line therapeutic drug, has been used to treat lung cancer, but GEM resistance poses a major limitation on the efficacy of GEM chemotherapy. Alantolactone (ALT), a sesquiterpene lactone compound isolated from Inula helenium, has been identified to exert anticancer activity in various types of cancer, including breast, pancreatic, lung squamous and colorectal cancer. However, the underlying mechanisms of the anticancer activity of ALT in lung cancer remain to be fully elucidated. The present study aimed to determine whether ALT enhances the anticancer efficacy of GEM in lung cancer cells and investigated the underlying mechanisms. The cell viability was assessed with a Cell Counting Kit-8 assay. The cell cycle, apoptosis and the level of reactive oxygen species (ROS) were assessed by flow cytometry, and the expression of cell cycle-associated and apoptosis-associated proteins were determined by western blot analysis. The results demonstrated that ALT inhibited cell growth and induced S-phase arrest and cell apoptosis in A549 and NCI-H520 cells. Furthermore, ALT increased the level of ROS, inhibited the Akt/glycogen synthase kinase (GSK)3β pathway and induced endoplasmic reticulum (ER) stress in A549 and NCI-H520 cells. Additionally, ALT treatment sensitized lung cancer cells to GEM. Analysis of the molecular mechanisms further revealed that ALT enhanced the anticancer effects of GEM via ROS-mediated activation of the Akt/GSK3β and ER stress pathways. In conclusion, combined treatment with ALT and GEM may have potential as a clinical strategy for lung cancer treatment.

Introduction

Lung cancer is one of the leading causes of cancer-associated mortality in China and globally (1). Lung cancer can be classified into non-small cell lung cancer (NSCLC), accounting for ~80-85% of cases, and small cell lung cancer (SCLC), accounting for ~10-15% of cases (2-4). At present, the standard treatment strategy for lung cancer includes surgical resection, chemotherapy and radiation therapy (5). Furthermore, chemotherapy remains one of the commonly used therapeutic regimens for advanced lung cancer (6). Gemcitabine (GEM) as a first-line therapeutic drug has been used to treat lung cancer, but GEM resistance poses a major limitation to the efficacy of GEM chemotherapy (7). Therefore, it is important to develop novel agents and therapeutic strategies to overcome resistance.

Numerous recent studies focused on natural products, which may be sources of novel natural antitumor agents (8-10). Furthermore, a number of antitumor drugs, including paclitaxel, docetaxel and vinorelbine, have been developed from natural products and are successfully used to treat cancer (11). It has been reported that certain natural products exert anticancer effects through a number of mechanisms of action, including the inhibition of phosphoinositide-3 kinase (PI3K)/Akt, induction of endoplasmic reticulum (ER) stress and the generation of reactive oxygen species (ROS) in various cancer types, including colorectal, lung and prostate cancer (12-14). Additionally, Wang et al (15) reported that licorice induces GEM-induced cytotoxicity by suppressing the Akt pathways in osteosarcoma cells. Cheng et al (16) reported that resveratrol enhances the sensitivity of pancreatic cancer cells to GEM via the accumulation of ROS.

Alantolactone (ALT), a sesquiterpene lactone compound isolated from Inula helenium (Fig. 1A), has been reported to exert anticancer effects against various types of cancer. ALT was demonstrated to promote apoptosis in colorectal cancer cells via ROS overproduction (17). ALT may induce apoptosis...
of human cervical cancer cells via ROS generation (18). In MDA-MB-231 breast cancer cells, ALT induces apoptosis via the ROS-mediated mitochondrion-dependent pathway (19). Furthermore, ALT may trigger apoptosis and induce cell cycle arrest in the G1/G0 phase in SK-MES-1 lung squamous cancer cells (20). Additionally, Maryam et al (21) reported that ALT enhanced the chemosensitivity of A549 cells to doxorubicin via ROS-mediated inhibition of signal transducer and activator of transcription 3 activation.

In the present study, it was first examined whether ALT may enhance the sensitivity of human lung cancer cells to GEM and then the underlying mechanisms were investigated. The results provide evidence that ALT in combination with GEM may be a promising strategy for treating lung cancer.

Materials and methods

Reagents and antibodies. ALT and N-acetyl-L-cysteine (NAC; a ROS scavenger) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-CCAAT-enhancer-binding protein homologous protein (CHOP) monoclonal antibody (cat. no. 5554), anti-phosphorylated (p) eukaryotic initiation factor 2α [p-eIF2α (Ser51); cat. no. 9271], anti-eIF2α (cat. no. 9722), anti-cyclin A2 (cat. no. 4656), anti-p-Akt (cat. no. 4058), anti-Akt (cat. no. 9272), anti-p-glycogen synthase kinase 3β [p-GSK3β (Ser9); cat. no. 5558], anti-GSK3β (cat. no. 12456), anti-β-actin (cat. no. 4967) and anti-caspase-3 (cat. no. 9662) were purchased from Cell Signal Technology, Inc. (Danvers, MA, USA). Anti-p21 (cat. no. 195720) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (cat. no. 7076) and HRP-conjugated anti-rabbit IgG (cat. no. 7074) were purchased from Cell Signal Technology Inc. Tunicamycin (TM; an ER stress agonist) was purchased from Merck KGaA, Darmstadt, Germany. Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO₂.

Cell viability assay. The A549 and NCI-H520 human lung cancer cell lines were provided by the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO₂.

Cell viability assay. The A549 and NCI-H520 cells were seeded in 6-well plates at a density of 2.5x10⁵ cells/well for 24 h at 37°C. Propidium iodide (PI) was used to stain the DNA in the samples for 15 min at 25°C, and flow cytometry was used to detect cell cycle distribution. The results were analyzed with FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA). The experiments were performed in triplicate.

Cell cycle analysis. The A549 and NCI-H520 cells were seeded in 6-well plates at a density of 2.5x10⁵ cells/well for 24 h at 37°C. Cell cycle analysis was measured with an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (cat. no. KGA104; Nanjing KeyGen Biotech Co., Ltd.), according to the manufacturer’s protocol. In brief, the A549 and NCI-H520 cells were pre-treated with NAC (8 mM) for 2 h at 37°C. The A549 cells were then treated with or without 4 µM ALT and 25 µM GEM for 24 h at 37°C. The NCI-H520 cells were treated with or without 40 µM ALT and 25 µM GEM for 24 h at 37°C. Annexin V-FITC and PI were used to stain the cells for 15 min at 37°C in dark room. The apoptotic cells were then detected with a FACScalibur flow cytometer and analyzed by FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA). The experiments were performed in triplicate.

Measurement of ROS generation. Intracellular ROS production was measured with a ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using flow cytometry, according to the manufacturer’s protocol. In brief, the A549 cells were treated with or without 4 µM ALT and 25 µM GEM for 12 h at 37°C. The NCI-H520 cells were treated with or without 40 µM ALT and 25 µM GEM for 12 h at 37°C. The cells were then incubated with 10 µM 2',7'-dichlorodihydrofluorescein diacetate for 15 min at 37°C in dark. The cells were analyzed using a FACScalibur flow cytometer. The results were analyzed with FlowJo 7.6 software and the experiments were performed in triplicate.

Western blot analysis. The A549 and NCI-H520 cells were seeded in 6-well plates at a density of 2.5x10⁵ cells/well for 24 h at 37°C. The cell cycle distribution was analyzed with a Cell Cycle Detection kit (cat. no. KGA512; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s protocols. In brief, the A549 cells were treated with or without 4 µM ALT and 25 µM GEM for 24 h at 37°C. The NCI-H520 cells were treated with or without 40 µM ALT and 25 µM GEM for 24 h at 37°C. Flow cytometry was used to detect cell cycle distribution. The results were analyzed with ModFit LT 3.0 (Verity Software House, Inc., Topsham, ME, USA). The experiments were performed in triplicate.
Figure 1. Effects of ALT and/or GEM treatment on the proliferation of lung cancer cells. (A) Chemical structure of ALT. (B) A549 and NCI-H520 cells were treated with various concentrations of ALT. (C) A549 and NCI-H520 cells were treated with various concentrations of GEM. (D) A549 cells were treated with ALT (4 μm) and/or GEM (25 μm). NCI-H520 cells were treated with ALT (40 μm) and/or GEM (25 μm). After 24 h of incubation, the cell viability was determined with a Cell Counting Kit-8 assay. (E) A549 cells were treated with ALT (4 μm) and/or GEM (25 μm). NCI-H520 cells were treated with ALT (40 μm) and/or GEM (25 μm). After 24 h of incubation, morphological changes were visualized under an inverted light microscope. Magnification, x200. Values are expressed as the mean ± standard deviation from three independent experiments. **P<0.01, ***P<0.001 and ****P<0.0001 vs. control. ++P<0.01 and +++P<0.001 vs. GEM. ###P<0.001 vs. ALT. GEM, gemcitabine; ALT, alantolactone.
2.5 x 10^5 cells/well for 24 h at 37°C. The A549 cells were treated with or without 4 µM ALT and 25 µM GEM for 12 h at 37°C. The NCI-H520 cells were treated with or without 40 µM ALT and 25 µM GEM for 12 h at 37°C. Furthermore, the A549 cells were treated with ALT (4 µm) and NCI-H520 cells were treated with ALT (40 µm) for 3, 6 or 12 h. In addition, the A549 and NCI-H520 cells were treated with LY294002 (4 µm) for 12 h. In a separate experiment, the A549 and NCI-H520 cells were pre-treated with NAC (8 mM) for 2 h at 37°C, followed by treatment with or without 4 µM ALT and 25 µM GEM for 12 h at 37°C for the A549 cells, and treatment with or without 40 µM ALT and 25 µM GEM for 12 h at 37°C for the NCI-H520 cells.

Following the different treatments, the A549 and NCI-H520 cells were harvested and lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) with protease inhibitors (Sigma Aldrich, St. Louis, MO, USA) on ice for 30 min and then centrifuged at 12,000 x g for 10 min at 4°C, as described previously (22). The proteins were then quantified with a Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein samples (40 µg protein/well) were separated by 10-15% SDS-PAGE, according to molecular weight of protein. Akt and p-Akt were separated by 10% SdS-PAGE, according to molecular weight of protein. It was notable that A549 cells significantly decreased the growth of A549 and NcI-H520 cells in a dose-dependent manner. It was observed that ALT, GEM and their combination notably increased the expression levels of activation of caspase-3, compared with those in the mono-treatment groups, the levels of activation of caspase-3 induced by ALT on the growth inhibition by GEM, the apoptotic rate was significantly increased in the combined group (Fig. 2A and B). The combination treatment with ALT and GEM significantly increased the S-phase arrest, compared with that by ALT or GEM treatment alone (Fig. 2A and B). Furthermore, western blot analysis was performed to examine the expression levels of S-phase-associated proteins in A549 and NCI-H520 cells. As depicted in Fig. 2C, ALT, GEM and co-treatment with ALT and GEM caused a notable upregulation of the expression levels of p21 but a downregulation of cyclin A2, compared with that in the control group. Compared with that in the mono-treatment groups, the levels of cyclin A2 in the co-treatment group were decreased, while the levels of p21 were increased in A549 and NCI-H520 cells. These results indicated that S-phase arrest contributes to the synergistic effect induced by ALT on the growth inhibition properties of GEM in A549 and NCI-H520 cells.

Results

ALT enhances GEM-induced cytotoxicity in A549 and NCI-H520 cells. To assess whether ALT synergizes with GEM to inhibit cell proliferation, A549 and NCI-H520 cells were first treated with increasing doses of ALT and GEM for 24 h, and the cell viability was assessed with a CCK-8 assay. As depicted in Fig. 1B and C, ALT or GEM significantly decreased the growth of A549 and NCI-H520 cells in a dose-dependent manner. It was notable that A549 cells [half maximal inhibitory concentration (IC₅₀)=6.63±1.0 µM] were more sensitive to ALT, compared with NCI-H520 cells (IC₅₀=59.91±1.16 µM), while the sensitivity of A549 cells (IC₅₀=8.39±1.02 µM) and NCI-H520 (IC₅₀=58.76±1.06 µM) to GEM was similar. Based on the results, 4 µM ALT and 25 µM GEM were used in A549 cells for the subsequent experiments. NCI-H520 cells were treated with 40 µM ALT and 25 µM GEM for the subsequent experiments. The cell viability of A549 and NCI-H520 cells was investigated with a CCK-8 assay. Furthermore, the results indicated that the viability of A549 and NCI-H520 cells was significantly decreased by combined treatment, compared with ALT or GEM treatment alone (Fig. 1D). Morphological observation also indicated that combined treatment notably decreased the percentage of surviving cells, compared with those subjected to ALT or GEM treatment alone (Fig. 1E). These results indicated that ALT significantly enhances the synergism of GEM by inhibiting the cell proliferation of A549 and NCI-H520 cells.

ALT enhances GEM-induced cell cycle arrest in A549 and NCI-H520 cells. Numerous studies demonstrated that cell cycle arrest induced by anticancer drugs has an important role in cell growth inhibition (23-25). To verify whether cell cycle arrest is involved in the GEM sensitization effect, the impact of ALT and GEM on the cell cycle distribution was determined in A549 and NCI-H520 cells with flow cytometry. The cell cycle assay demonstrated that ALT, GEM and their co-treatment significantly induced S-phase arrest, compared with the control group (Fig. 2A and B). The combination treatment with ALT and GEM significantly increased the S-phase arrest, compared with that caused by ALT or GEM treatment alone (Fig. 2A and B). Furthermore, western blot analysis was performed to examine the expression levels of S-phase-associated proteins in A549 and NCI-H520 cells. As depicted in Fig. 2C, ALT, GEM and co-treatment with ALT and GEM caused a notable upregulation of the expression levels of p21 but a downregulation of cyclin A2, compared with that in the control group. Compared with that in the mono-treatment groups, the levels of cyclin A2 in the co-treatment group were decreased, while the levels of p21 were increased in A549 and NCI-H520 cells. These results indicated that S-phase arrest contributes to the synergistic effect induced by ALT on the growth inhibition properties of GEM in A549 and NCI-H520 cells.

ALT enhances GEM-induced apoptosis in A549 and NCI-H520 cells. To further investigate the synergistic effect induced by ALT on the growth inhibition by GEM, the apoptosis of A549 and NCI-H520 cells treated with ALT and GEM was examined with flow cytometry. As depicted in Fig. 3A and B, ALT, GEM and their combination significantly increased the apoptotic rate of A549 and NCI-H520 cells. Compared with that in the mono-treatment groups, the apoptotic rate was significantly increased in the combined group (Fig. 3A and B). The levels of the apoptosis-associated protein caspase-3 in A549 and NCI-H520 cells were also examined with western blot analysis. It was observed that ALT, GEM and their combination notably increased the expression levels of activation of caspase-3. Compared with those in the mono-treatment groups, the levels of activation of caspase-3 were notably increased in the combined treatment group.
(Fig. 3C). These results demonstrated that ALT significantly enhances the sensitivity to GEM by inducing cell apoptosis in A549 and NCI-H520 cells.

ALT sensitizes GEM-mediated cell apoptosis via ROS production in A549 and NCI-H520 cells. Previous studies indicated that ROS has a critical role in anticancer drug-mediated apoptosis (26,27). Therefore, the effects of ALT and GEM on ROS production in A549 and NCI-H520 cells were assessed by flow cytometry. As depicted in Fig. 4A and B, ALT, GEM, and their combination significantly increased ROS accumulation in A549 and NCI-H520 cells. Compared with the mono-treatments, the co-treatment significantly enhanced ROS accumulation. To further investigate whether ROS generation contributes to ALT- and GEM-mediated apoptosis, A549 and NCI-H520 cells were pre-treated with NAC (ROS scavenger) prior to treating the cells with ALT and GEM, and apoptosis was then detected by flow cytometry. The results indicated...
that pre-treatment with NAC significantly decreased ALT- and GEM-induced apoptosis in A549 and NCI-H520 cells (Fig. 4C and D). Additionally, western blot analysis revealed that pre-treatment with NAC notably attenuated the increases in the levels of activation of caspase-3 induced by ALT and GEM treatment in A549 and NCI-H520 cells (Fig. 4E). Thus, these results demonstrated that ALT enhances GEM-mediated apoptosis via increasing the intracellular ROS production in A549 and NCI-H520 cells.

ALT sensitizes A549 and NCI-H520 cells to GEM-mediated cell apoptosis via induction of ROS-mediated ER stress. It has been reported that ROS generation induces cell apoptosis through activating ER stress pathways in a variety of cancer
Figure 4. Effects of ALT and/or GEM on intracellular ROS generation in lung cancer cells. (A) A549 cells were treated with ALT (4 µm) and/or GEM (25 µm). NCI-H520 cells were treated with ALT (40 µm) and/or GEM (25 µm). After 12 h of incubation, the levels of ROS were examined by flow cytometry. (B) Bar graphs present the quantification of the levels of ROS. A549 and NCI-H520 cells were pre-treated with NAC (8 mM) for 2 h, followed by treatment with ALT (4 µm) and/or GEM (25 µm) in A549 cells and treatment with ALT (40 µm) and/or GEM (25 µm) in NCI-H520 cells for 24 h. (C) Cell apoptosis was examined by flow cytometry.
cell types, including prostate, bladder and lung cancer (28-30). To confirm whether ER stress is involved in ROS-mediated apoptosis induced by the test drugs, the effect of ALT and GEM on hallmarks of ER-associated apoptosis, including eIF2α phosphorylation and CHOP expression, was determined in A549 and NCI-H520 cells with western blot analysis. As indicated in Fig. 5A, the levels of eIF2α phosphorylation and CHOP expression were notably unregulated by ALT in A549 and NCI-H520 cells. Combination treatment with ALT and GEM notably increased the phosphorylation of eIF2α and the expression of CHOP, compared with that caused by each drug alone (Fig. 5B). Furthermore, A549 and NCI-H520 cells were treated with ALT and GEM in the presence of NAC and the phosphorylation of eIF2α and the expression of CHOP were then measured. It was revealed that NAC attenuated the phosphorylation of eIF2α and the expression of CHOP induced by ALT and GEM (Fig. 5C). To further confirm whether ER stress is involved in GEM-mediated growth inhibition, the effects of TM, an ER stress agonist, on GEM-induced growth inhibition of A549 and NCI-H520 cells were assessed. As depicted in Fig. 5D, combination treatment with TM and GEM significantly decreased the cell viability compared with GEM alone. Collectively, these results revealed that ALT increases GEM-mediated apoptosis via ROS-mediated ER stress activation.

ALT sensitizes GEM-mediated cell apoptosis via ROS-mediated inhibition of the Akt/GSK3β pathway in A549 and NCI-H520 cells. The Akt pathway is an important pathway associated with ROS-mediated apoptosis in various cancer cell types, including bladder, lung and pancreatic cancer (31-34). To determine whether the Akt pathway is involved in ROS-mediated apoptosis, the effects of ALT and GEM on the levels of the expression of Akt, GSK3β, p-Akt and p-GSK3β in A549 and NCI-H520 cells were determined by western blot analysis. As depicted in Fig. 6A, the levels of p-Akt and p-GSK3β (Ser 9) were notably decreased by ALT, but the levels of total Akt and GSK3β were not notably affected. Furthermore, combination treatment notably inhibited the levels of p-Akt and p-GSK3β, compared with the mono-treatment groups (Fig. 6B). In another experiment, A549 and NCI-H520 cells were treated with LY294002, a PI3K inhibitor, and the levels of p-GSK3β and p-Akt were detected with western blot analysis. As depicted in Fig. 6C, LY294002 notably decreased the levels of p-GSK3β and p-Akt in A549 and NCI-H520 cells. To further determine the role of the Akt pathway in ROS-mediated cell apoptosis, A549 and NCI-H520 cells were pre-treated with NAC followed by the test drugs, and the effect on the levels of p-Akt was determined. As depicted in Fig. 6D, pre-treatment with NAC reduced the effect of co-treatment with ALT and GEM on p-Akt. The effect of pre-treatment with LY294002 on cell growth inhibition induced by GEM was then assessed. As depicted in Fig. 6E, LY294002 pre-treatment significantly increased the GEM-induced inhibition of A549 and NCI-H520 cell growth. Collectively, these results indicated that ALT increases GEM-mediated apoptosis via ROS-mediated activation of the Akt/GSK3β pathway.

Discussion

GEM, as a first-line chemotherapeutic drug, is frequently used for the treatment of a number of cancer types, including lung
Figure 5. ALT enhances the anticancer activity of GEM in lung cancer cells via the reactive oxygen species-mediated endoplasmic reticulum stress pathway. 

(A) A549 cells were treated with ALT (4 µm) and NCI-H520 cells were treated with ALT (40 µm) for 3, 6 or 12 h. (B) A549 and NCI-H520 cells were treated with ALT (4 or 40 µm) and/or GEM (25 µm) for 12 h. (C) A549 and NCI-H520 cells were pre-treated with NAC (8 mM) for 2 h, followed by treatment with ALT (4 or 40 µm) and GEM (25 µm) for 12 h. p-eIF2α and CHOP were determined by western blot analysis and β-actin served as a loading control.
or GEM alone, their combination caused a notable upregulation in the expression of cyclin A2. Furthermore, compared with ALT in the expression of cyclin-dependent kinase inhibitor p21 and a revealed that ALT alone or GEM alone caused an upregulation and NcI-H520 cells. The combination of the two drugs demonstrated that ALT and GEM induce apoptosis in A549 and NcI-H520 cells. The present results indicated that ALT enhances GEM-induced cell apoptosis by activation of caspase-3 in A549 and NCI-H520 cells. Cell apoptosis-associated protein analysis also revealed that the drug combination notably increases the level of activation of caspase-3. These results indicated that ALT enhances GEM-induced cell apoptosis by activation of caspase-3 in A549 and NCI-H520 cells.

High levels of ROS have been documented to induce apoptosis in various cancer types, including bladder, lung and cervical cancer, which have a notable role in cell apoptosis induced by anticancer drugs (29,34,49). Previous research demonstrated that ALT induces cell apoptosis in various cancer types, including colorectal, cervical and breast cancer, via increasing the generation of ROS (17-19). Maryam et al (21) demonstrated that ALT enhances the chemosensitivity of A549 lung adenocarcinoma cells to doxorubicin via ROS generation. Cheng et al (16) also reported that resveratrol enhances the sensitivity of pancreatic cancer cells to GEM via inducing the accumulation of ROS. In the present study, an increase in ROS generation was observed in ALT- or GEM-treated A549 and NCI-H520 cells. Compared with ALT or GEM alone, their combination significantly increased the accumulation of A549 and NCI-H520 cells in the S phase. Additionally, cell cycle-associated protein analysis revealed that ALT alone or GEM alone caused an upregulation in the expression of cyclin-dependent kinase inhibitor p21 and a downregulation of cyclin A2. Furthermore, compared with ALT or GEM alone, their combination caused a notable upregulation in the expression of p21 and downregulation of cyclin A2. These results indicated that ALT enhanced the anti-proliferative effect of GEM in A549 and NCI-H520 cells via p21 and cyclin A2-mediated S-phase arrest.

Apoptosis is an important cellular process and numerous anticancer drugs, including paclitaxel, doxorubicin, carboplatin and curcumin, prevent tumor progression via inducing cell apoptosis (45-47). It is well known that ALT and GEM induce apoptosis in lung cancer (21,48). The present results demonstrated that ALT and GEM induce apoptosis in A549 and NCI-H520 cells. The combination of the two drugs significantly increased the rate of apoptosis of A549 and NCI-H520 cells, compared with that achieved with each drug alone. Cell apoptosis-associated protein analysis revealed that ALT or GEM may induce cell apoptosis by activation of caspase-3 in A549 and NCI-H520 cells. Cell apoptosis-associated protein analysis also revealed that the drug combination notably increases the level of activation of caspase-3. These results indicated that ALT enhances GEM-induced cell apoptosis by activation of caspase-3 in A549 and NCI-H520 cells.
adenocarcinoma cells to doxorubicin via the ROS-mediated ER stress apoptosis pathway. Consistent with this, the present study indicated that ALT caused a notable deregulation of ER stress-associated proteins, including increases in eIF2α phosphorylation and CHOP expression in A549 and NCI-H520 cells. Combination treatment with ALT and GEM notably increased the phosphorylation of eIF2α and CHOP expression, compared with that obtained with each drug alone. Furthermore, inhibition of ROS generation by NAc abrogated the ALT- and GEM-induced ER stress activation in A549 and NCI-H520 cells. Additionally, combination treatment with TM significantly enhanced the effect of GEM to decrease the viability of lung cancer cells. Collectively, these results indicated that ALT enhances GEM-mediated apoptosis via the ROS-mediated, ER stress-induced apoptosis pathway.

The Akt pathway is involved in regulating cell survival and death (56). Therefore, inhibition of the Akt signaling pathway has been considered an effective approach for the treatment of human cancer types, including prostate and gastric cancer (57,58). It has been reported that the inhibition of Akt induced cancer cell apoptosis via inhibition of various downstream targets, including inhibition of the phosphorylation of GSK3β at Ser9 (59,60). Furthermore, evidence indicated that increased ROS generation induced by anticancer drugs triggered cell apoptosis via inhibition of the Akt signaling pathway (31,61). Li et al (62) reported that phenoxodiol enhances the antitumor activity of GEM against gallbladder cancer through suppressing the Akt pathway. Furthermore, Tuya et al (63) reported that trichosanthin enhances the antitumor effect of GEM via inhibition of the Akt pathway in NSCLC. In the present study, it was confirmed that ALT reduces the levels of p-Akt and p-GSK3β in A549 and NCI-H520 cells. Combination treatment of ALT and GEM notably reduced the levels of p-Akt and p-GSK3β, compared with that in the mono-treatment groups. Furthermore, pre-treatment of NAC efficiently abrogated the combination treatment-induced reduction in the levels of p-Akt and p-GSK3β. Additionally, LY294002 decreased the levels of...
p-GSK3β and increased GEM-induced cell growth inhibition in A549 and NCI-H520 cells. These results indicated that ALT enhances GEM-mediated apoptosis via ROS-mediated inhibition of the Akt/GSK3β pathway.

In conclusion, the present study demonstrated that ALT enhances the antitumor efficacy of GEM via the ROS-mediated inhibition of the Akt/GSK3β pathway and activation of the ER stress pathway in A549 and NCI-H520 cells. These results indicated that the combination of ALT and GEM may provide a potential clinical strategy for lung cancer treatment. Furthermore, these results will be further validated in in vivo experiments.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JiqW and JiaW conducted the experiments and analyzed the data. JiqW made substantial contributions to the design of the present study and prepared the manuscript. YZ, XL, JiqW, BL and YL performed the western blotting and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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