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Original Research

Raddeanin A suppresses lung cancer cell proliferation via induction of apoptosis and increased production of ROS

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Abstract: At present, in vitro cell experiments have confirmed that RaddeaninA can effectively inhibit the proliferation of some tumor cells, but the effect of RaddeaninA on lung cancer cells has not been observed. Therefore, this study explored its effect on lung cancer cells and its mechanism of action. Human lung cancer cell lines were treated with serum-free medium and varied concentrations of Raddeanin A. Cell proliferation and apoptosis were determined using MTT, and flow cytometric assays, respectively. The intracellular level of ROS was determined using DCFH-DA assay. Protein and mRNA expressions of bax, bcl-2 and cyt c were measured using Western blotting and qRT-PCR. RaddeaninA treatment can promote PC-9 cell apoptosis in a time and dose-dependent manner ($p<0.05$). Treatment of PC-9 cells with Raddeanin significantly and dose-dependently increased the activities of caspase-9 and caspase-3 ($p<0.05$), and led to significant and dose-dependent increases in ROS levels ($p<0.05$). Treatment of PC-9 cells with Raddeanin A led to significant and dose-dependent decreases in mitochondrial membrane potential ($p<0.05$). It significantly and dose-dependently upregulated bax mRNA and protein expressions, but down-regulated bcl-2 mRNA and protein expressions significantly and dose-dependently ($p<0.05$). On the other hand, Raddeanin significantly and dose-dependently down-regulated cytoplasmic bax protein expression, while upregulating cyt c expression ($p<0.05$). Similarly, bax protein expression was significantly and dose-dependently upregulated in mitochondria, but the corresponding cyt c expression was significantly and dose-dependently down-regulated ($p<0.05$). Raddeanin A is a potential and effective lung cancer chemotherapy drug, which can induce lung cancer cell apoptosis and inhibit proliferation.

Key words: Lung cancer; Proliferation; Apoptosis; Raddeanin A.

Introduction

Lung cancer, the most common malignant tumor in the lungs, is characterized by high mortality and morbidity (1). In 2018, lung cancer accounted for about 25% of all cancer deaths in the United States. Treatments for lung cancer vary but may include surgery, chemotherapy, radiation therapy, targeted drug therapy and immunotherapy. The combination of surgery and postoperative chemotherapy offers the best treatment. Long-term survival of patients with advanced lung cancer cannot be effectively treated with available strategies, since chemoresistance remains a militating factor (2). This has necessitated the search for novel strategies/approaches that can effectively treat the disease.

Synthetic drugs are often toxic and characterized by adverse reactions, but herbs used in Traditional Chinese Medicine (TCM) have been reported to be less toxic and have shown promise in mitigating multi-drug resistance (3, 4). For instance, taxol, a diterpenoid alkaloid isolated from the bark of gymnosperm *Taxus chinensis* and *pacific yew*, is an antitumor agent. It is widely used for the treatment of various tumors such as lung, head, neck, and breast cancers (5-7).

Radde Anemone Rhizome is the dried rhizome of the sea anemone raddeana Regel, which has been shown to have significant anti-inflammatory, analgesic, anticonvulsant and sedative effects. Radde Anemone Rhizome contains triterpene saponins, volatile oils, lactones, polysaccharides and other active ingredients. Among them, Raddeanin A is a high content of oleic acid triterpenoids in Radde Anemone Rhizome, and it is a monomeric saponin with powerful anti-tumor activity. Raddeanin A, as a newly discovered anti-tumor monomer drug, has attracted widespread attention. The occurrence and development of tumors is an extremely complicated process. Therefore, the anti-cancer mechanism of Raddeanin A is also very complicated. It has not been fully understood by people, and there are few clinical application studies. At present, studies have shown that Raddeanin A induces apoptosis and cell cycle arrest and inhibits invasion, migration and angiogenesis in malignant cell lines and preclinical models (8-10). It inhibits the proliferation of osteosarcoma cells via the ROS/JNK and NF-κB signaling pathways (11). It has been shown to promote apoptosis in colon cancer cells via the down-regulation of Wnt/β-catenin and NF-κB signaling pathways.

Guan YD and other studies found that Raddeanin A can inhibit the proliferation of breast cancer cells (T47D, MCF-7 and MDA-MB-231) in vitro, and can also induce breast cancer cell apoptosis and autophagy, which is positively correlated with the dose (12). Peng Z found that RA can induce apoptosis by inhibiting the

STAT3/NFL3 signaling pathway, thereby reversing the chemotherapy resistance of choriocarcinoma (13). Li JN confirmed that RA can block the liver cancer cell QGY-7703 in the G0/G1 cycle, inhibit its division and induce cell apoptosis, and enhance the anti-tumor effect of cisplatin (14). However, it is not clear whether Raddeanin A and associated compounds inhibit lung cancer cell proliferation. The present study investigated the effect of Raddeanin A on lung cancer cell proliferation, and the mechanism involved.

Materials and Methods

Materials

Human lung cancer cell lines (A549, PC-9, H1299 and H358) were obtained from the Wuhan cell bank of the Chinese Academy of Sciences. Annexin V-FITC/PI apoptosis detection kit, MTT assay kit, Hoechest33342 staining solution, and ROS assay kit were purchased from Beijing Beyotime Biotechnology Co. Ltd. Caspases-8, -9 and -3 assay kits were bought from Jiangsu Kaiji Biotechnology Co. Ltd., while Raddeanin A was obtained from Selleck Co. Ltd. Total RNA extraction and RT-PCR kits were purchased from Takara Corporation (Japan), while SYBR Green reagent was a product of ABI Co. Ltd. (USA). Rabbit anti-β-actin, bax, bcl-2, cyt c, COX IV primary antibodies and horseradish peroxidase-labeled second antibody were products of CST Co. Ltd. Fetal bovine serum (FBS), RPMI-1640 medium, DMEM, and double antibody were obtained from Gibco Co. Ltd. (USA). Inverted microscope (ECLIPSE MA100) was purchased from Nikon (Japan); a flow cytometer was obtained from BD Co. Ltd. (USA), while RT-PCR machine (BJ001271) was a product of ABI (USA).

Cell culture

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37°C for 24h in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced with a fresh one every two days. After 1 week of incubation, the adherent confluent cells were trypsinized with 0.25% trypsin-EDTA (2 mL), cultured again and passaged for later use. When the cells attained 60-70% confluency, they were treated with serum-free medium and varied concentrations of Raddeanin A (0.5-8.0μM) for 24h. Normal cell culture without Raddeanin A served as the control group. Cells in the logarithmic growth phase were selected and used for this study.

Cell proliferation assay

Cell proliferation is measured by 3-(4,5-dimethyl-th iazol-2-yl)-2,5-diphenyl-2H-

tetrazolium bromide (MTT). The cells were seeded into 24-well plates at a density of 2×10^8 cells/well and cultured in DMEM for 24 h. Raddeanin A (0.5-8.0μM) was added to the cells and incubated for 3 days. At the end of the third day, 20μl of 5g/L MTT solution was added to the wells, followed by incubation for another 4h. The medium was finally replaced with 150mL of 0.1% dimethyl sulfoxide (DMSO) solution, agitated at 50 oscillations/min for 10min to solubilize the formazan crystals, and absorbance of each well was read in a microplate reader at 540nm. The degree of proliferation was determined at different time points: 24, 48 and 72h. Cell proliferation was calculated as shown in Equation 1:

$$Cell\ proliferation\ (\%) = \frac{Absorbance\ of\ the\ experimental\ group \times 100}{Absorbance\ of\ the\ control\ group} \quad (1)$$

Apoptosis assay

The PC-9 cells were seeded at a density of 2.5×10^6 cells/well in 6-well plates and cultured at 37°C for 72h. They were thereafter washed with phosphate-buffered saline (PBS), and thoroughly mixed with 500μL of binding buffer. Then, the cells were stained with 10μL each of Annexin V-fluorescein isothiocyanate and propidium iodide (PI) within 10min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 488 nm.

Determination of reactive oxygen species (ROS) level

The levels of ROS in PC-9 cells were determined using DCFH-DA assay. The cells treated with Raddeanin A (1.0-4.0μM) were washed with PBS after an initial incubation for 72h. Then, a 10μM solution of DCFH-DA was added to the plates and incubated for another 35 min at 37˚C. Thereafter, the cells were washed with PBS and injected into the flow cytometer for analysis.

Determination of mitochondrial membrane potential in lung cancer cells

The mitochondrial membrane potential of PC-9 cells was determined to flow cytometrically using the assay kit with JC-1 as a fluorescent probe.

Determination of caspase-8, caspase-9 and caspase-3 activities in lung cancer cells

Lung cancer cells (PC-9) treated with varying concentrations of Raddeanin A (1.0-4.0μM) were lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer. The protein concentration was determined using the bicinchoninic acid (BCA) protein quantification method. The activities of caspases-8, -9 and -3 were determined in cell lysates (150μg protein) using their respective enzyme-linked immunosorbent assay (ELISA) kits.

Real-time quantitative polymerase chain reaction(qRT-PCR)

The mRNA expressions of bax and bcl-2 were determined using qRT-PCR. A total RNA extraction kit was used to extract total RNA from PC-9 cells. The qRT-PCR was carried out using the standard method, and cDNA was obtained via reverse transcription. The RT-PCR was performed using SYBR Green dye method, while the relative expressions of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal reference gene (15). The primer sequences used are shown in Table 1.

Western blotting

The PC-9 cells (5×10^8 cells/L) were incubated with Raddeanin for 24h. The cells were then washed twice with PBS and lysed with 250μL of ice-cold RIPA buffer containing protease and phosphatase inhibitors. The

Table 1. Primer sequences used for qRT-PCR.

Gene	Sequence
Bax	Forward: 5'-TGCTTCAGGGTTTCATCCAG-3' Reverse: 5'-AACATTTCAGCCGCCACTC-3'
Bcl-2	Forward: 5'-TTCGCCGAGATGTCCAGTCAGC-3' Reverse: 5'-GTTGACGCTCTCCACACACA-3'
GAPDH	Forward: 5'-TGCGATTACGCTTTCGCAGC-3' Reverse: 5'-CCAGCAGGCAGATGCGT-3'

resultant lysate was centrifuged at 14,000rpm for 20min at 4°C, and the protein concentration of the supernatant was determined using the BCA method. A portion of total cell protein (50μg) from each sample was separated on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110V and 90°C for 120 min. Subsequently, non-fat milk powder (5%) in Tris-buffered saline containing 0.2% Tween-20 (TBS-T) was added with gentle shaking at 37°C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4°C with primary antibodies of bax, bcl-2, cytochrome c(cyt c), Cox IV and β-actin, each at a dilution of 1 to 2000. Then, the membrane was washed thrice with PBS and further incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 2h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced Chemiluminescence (ECL). The respective protein expression levels were normalized to that of β-actin which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS (20.0). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p<0.05$.

Results

Effect of Raddeanin A on lung cancer cell proliferation

As shown in Figure 1, Raddeanin A treatment significantly inhibited the proliferation of lung cancer cells time- and dose-dependently ($p < 0.05$). The PC-9 cells were most sensitive to Raddeanin A treatment.

Effect of Raddeanin A on lung cancer cell apoptosis

Normal cell nucleus was uniformly colored and appeared spherical or fusiform, but was condensed and smaller after treatment with Raddeanin A (Figure 1). Raddeanin A treatment significantly and dose-dependently promoted PC-9 cell apoptosis ($p<0.05$; Figure 2).

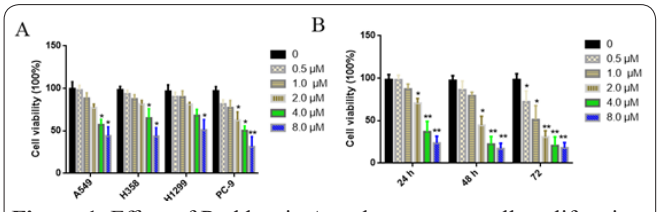

Figure 1. Effect of Raddeanin A on lung cancer cell proliferation. (A): Lung cancer cell proliferation after 12-h treatment; (B): Proliferation of PC-9 cells at 24, 48 and 72h post-treatment. *$p<0.05$; **$p<0.01$, compared with control group.

Levels of apoptotic markers in lung cancer cells

Treatment of PC-9 cells with Raddeanin A produced no significant effect on caspase 8 activity ($p>0.05$), but significantly and dose-dependently increased the activities of caspase-9 and caspase-3 ($p<0.05$; Figure 3).

Effect of Raddeanin A on ROS levels in lung cancer cells

Treatment of PC-9 cells with Raddeanin A led to significant and dose-dependent increases in their ROS levels ($p<0.05$). These results are shown in Figure 4.

Effect of Raddeanin A on NAC-induced ROS clearance

N-Acetyl-L-cysteine (NAC) treatment significantly reversed the inhibitory effect of Raddeanin A on PC-9 cell proliferation but significantly inhibited caspase-3

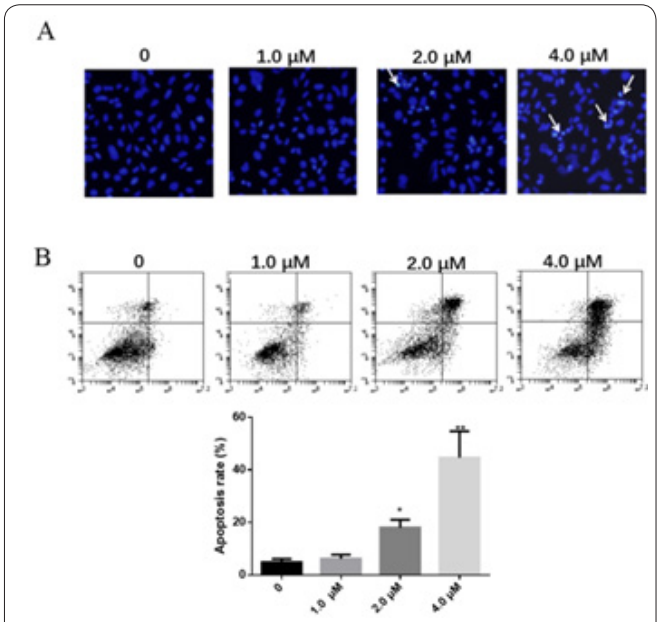

Figure 2. Effect of Raddeanin A on lung cancer cell apoptosis. (A): Morphology of PC-9 cell nucleus after 24h of Raddeanin A treatment, as revealed by fluorescence microscope; (B): PC-9 cell apoptosis as determined using a flow cytometer. *$p<0.05$; **$p<0.01$, compared with control cells.

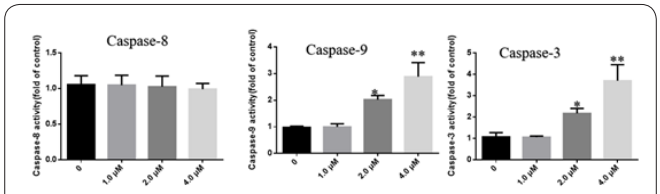

Figure 3. Effect of Raddeanin A on levels of apoptotic markers in lung cancer cells. *$p<0.05$; **$p<0.01$, compared with the control group.

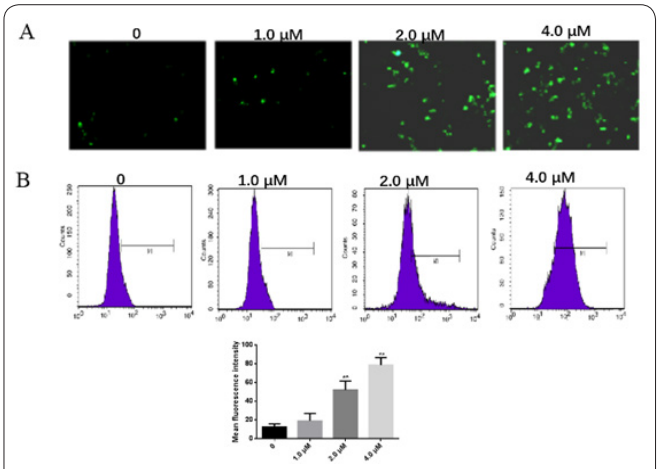

Figure 4. Effect of Raddeanin A on ROS levels in lung cancer cells. (A): Levels of ROS in PC-9 cells, as observed using a fluorescence microscope; (B): levels of ROS in PC-9 cells, as determined using flow cytometer. *p<0.05. **p<0.01, when compared with control cells.

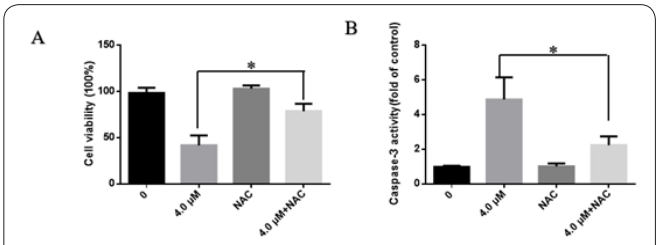

Figure 5. Effect of Raddeanin A on NAC-induced ROS clearance. *p<0.05 compared with the control group.

activity (p<0.05; Figure 5).

Effect of Raddeanin A on mitochondrial membrane potential

As shown in Figure 6, the treatment of PC-9 cells with Raddeanin A led to significant and dose-dependent decreases in their mitochondrial membrane potential (p<0.05).

Effect of Raddeanin A on bax, bcl-2 and cyt c protein levels

Raddeanin A treatment significantly and dose-dependently upregulated bax mRNA and protein expressions, but down-regulated bcl-2 mRNA and protein expressions significantly and dose-dependently (p<0.05). It also significantly and dose-dependently down-regulated cytoplasmic bax protein expression, while upregulating cyt c expression (p<0.05). Similarly, bax protein expression was significantly and dose-dependently upregulated in mitochondria, but the corresponding cyt c expression was significantly and dose-dependently down-regulated (p<0.05). These results are shown in Figure 7.

Discussion

Rhizoma Anemones Raddeanae (RAR) is the dry rhizome of *Anemone raddeana* Regel, which belongs to the *Ranunculaceae* family. It is used in TCM to treat rheumatism, wind and cold symptoms, spasms, joint pain and ulcer (16). This drug was widely used in ancient China to facilitate urination and treat intestinal obstruction (16). Raddeanin A possesses anti-inflammatory and antitumor effects. It promotes proteasome-mediated degradation and inhibition of androgen receptor (AR) gene transcription. It has been reported to promote the growth-inhibitory effect of docetaxel, a drug used in prostate cancer chemotherapy (17). Bone metastasis is a serious complication of advanced breast cancer, resulting in osteolysis and increased mortality. Studies have shown that Raddeanin A inhibited osteoclast formation and bone resorption, and suppressed osteolysis

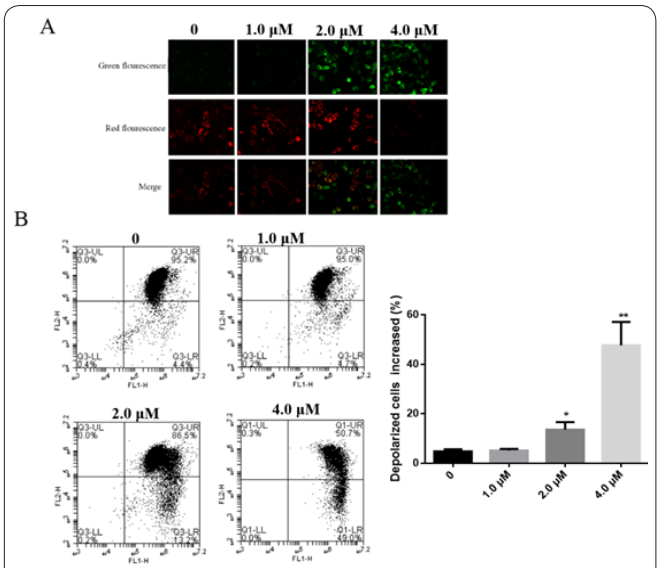

Figure 6. Effect of Raddeanin A on mitochondrial membrane potential. (A): Mitochondrial membrane potential of PC-9 cell as observed with a fluorescence microscope. Red fluorescence represents normal mitochondrial membrane potential, while green stands for decreased mitochondrial membrane potential. (B): Mitochondrial membrane potential of PC-9 cell as determined using flow cytometer. *p<0.05; **p<0.01, compared with control group.

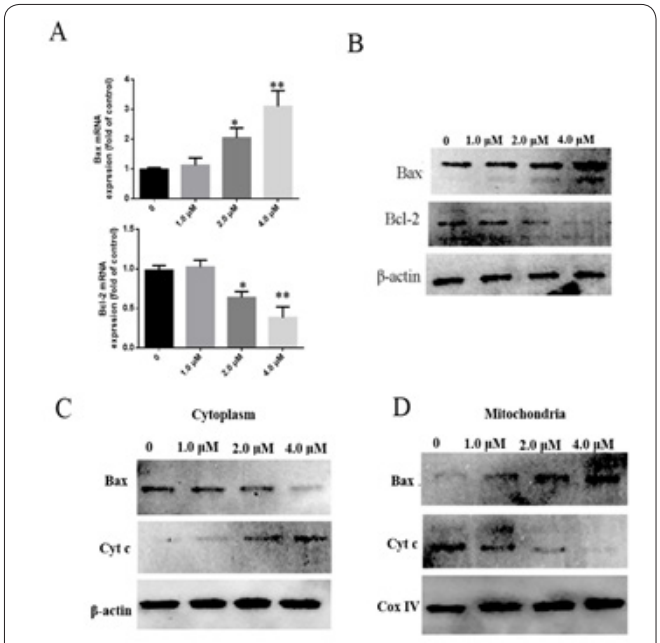

Figure 7. Effect of Raddeanin A on bax, bcl-2 and cyt c protein levels. (A): Levels of expressions of bax and bcl-2 mRNAs as measured using qRT-PCR; (B): Protein expressions of bax and bcl-2 as determined using Western blotting; (C): Levels of expression of bax and cyt c proteins in the PC cell cytoplasm; and (D): Protein expressions of bax and cyt c in mitochondria. *p<0.05; **p<0.01, compared with control group.

in mouse calvarial defect model via a mechanism involving the SRC/AKT signaling pathway. Thus, Raddeanin A may be a potential candidate drug for breast cancer-induced osteolysis (18). It has also been reported to promote apoptosis in gastric cancer cells (19). In one study, it was reported that Raddeanin A significantly inhibited the proliferation, migration and tubule-forming ability of HCT-15 cells. Molecular studies have shown that Raddeanin A inhibits the phosphorylation of vascular endothelial growth factor receptor (VEGFR2) and its downstream protein kinases, suggesting that it may inhibit tumor angiogenesis (20). Results of previous studies reviewed to date suggest that Raddeanin A may have the potential for use in cancer treatment, although the precise molecular mechanism has not been fully elucidated.

Apoptosis, also known as programmed cell death, or "cellular suicide", is an orderly process in which cell contents are packaged into small packets of membrane for "garbage collection" by immune cells. The biochemical events that culminate in apoptosis include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA degradation. It is one of the main anti-tumor mechanisms of chemotherapeutic drugs. Most anticancer drugs currently used in clinical oncology engage the two major apoptotic signaling pathways to trigger cancer cell death (21). The present study investigated the effect of Raddeanin A on lung cancer cell proliferation, and the mechanism involved. The results showed that Raddeanin A significantly and dose-dependently inhibited lung cancer cell proliferation, with PC-9 cells being the most sensitive to inhibition. These results indicate that Raddeanin A may inhibit the growth of lung cancer cells, and are in agreement with those of previous reports. Similarly, Hoechest33342 fluorescence staining showed that Raddeanin A promoted nuclear condensation of PC-9 cells. Nuclear condensation is one of the hallmarks of apoptosis. The anti-proliferative effect of Raddeanin A likely proceeds via induction of apoptosis. Raddeanin A has been reported to inhibit the growth and promote apoptosis of HCT-116 cells via a mechanism involving the down-regulation of the PI3K/AKT signal transduction pathway (22). It promoted HCT-116 cell cycle arrest at the G_0/G_1 phase since cyclins D_1 and E protein expressions were significantly down-regulated in the presence of Raddeanin A (22). Raddeanin A has been shown to significantly and dose-dependently inhibit the growth and promote apoptosis of gastric cancer cells (BGC-823, SGC-7901 and MKN-28) at different differentiation stages (23).

Reactive oxygen species (ROS), important intracellularly active molecules, are involved in different regulatory processes of cells (13). At low concentrations, ROS stimulate signal transmission, but poison cells at high concentration via apoptosis (24). Raddeanin A induced apoptosis in glioma cells through ROS/JNK signal transduction pathway, while NAC ameliorated Raddeanin A-induced apoptosis by scavenging ROS (25). N-Acetyl-L-cysteine (NAC)-induced ROS clearance has been reported in many cancer studies. N-Acetyl-L-cysteine (NAC) reversed

Raddeanin A-induced inhibition of proliferation of epithelial ovarian cancer cells (Skov3). Pretreatment of tumor cells with NAC promoted cell cycle arrest but had no significant effect on Raddeanin A-induced apoptosis (26). In this study, the treatment of PC-9 cells with Raddeanin A led to significant and dose-dependent increases in their ROS levels. However, NAC treatment significantly reversed the inhibitory effect of Raddeanin A on PC-9 cell proliferation but inhibited caspase-3 activity significantly. Raddeanin A-induced lung cancer cell apoptosis likely proceeds via increases in ROS levels. Increased ROS levels lead to the upregulation of bax expression and translocation to the mitochondrial membrane where it dimerizes to activate mitochondrial apoptosis pathways. N-Acetyl-L-cysteine (NAC) on the other hand, partially reversed the upregulated expression of bax and decreased the mitochondrial membrane potential induced by Raddeanin A. The results of this study are similar to those obtained in a previous study, where it was reported that Raddeanin A significantly inhibited the metastasis of osteosarcoma cells and enhanced ROS levels, leading to decreased mitochondrial membrane potential (11). Raddeanin A also significantly increases the toxicity of other chemotherapy drugs. For instance, it increased the sensitivity of bile duct cancer cells to 5-fluorouracil (5-Fu) treatment, thereby potentiating the effect of 5-Fu and down-regulating the expression of Wee1 protein (27). Chemoresistance is a major problem in the treatment of choriocarcinoma. A study has shown that Raddeanin A reduced drug resistance index of resistant cells, activated caspase-3-dependent apoptosis, and inhibited the activation of the STAT3/NFIL3 pathway (13). In addition, inhibition of eEF-2K-mediated autophagy enhanced *in vitro* cytotoxicity of Raddeanin A to human breast cancer cells (12). Inhibition of STAT3 phosphorylation promoted apoptosis of human osteosarcoma cells, enhanced the sensitivity of the cells to chemotherapy drugs, improved clinical effectiveness and reduced toxicity of antitumor drugs (28).

The results obtained in this study suggest that Raddeanin A suppresses lung cancer cell proliferation via induction of apoptosis and increased production of ROS. Thus, Raddeanin A offers great promise as an effective drug or potential candidate for improving lung cancer chemotherapy.

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Conflicts of interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yusheng Shu*, Hongcan Shi*; Weiguo Jin, Shichun Lu, Xiaolin Wang, Yusheng Shu*, Hongcan Shi* collected and analysed the data; Weiguo Jin, Shichun Lu wrote the text and all authors have read and approved the text before publication.
Weiguo Jin and Shichun Lu contributed equally to this work as co-first author. Yusheng Shu* and Hongcan Shi* are the co-corresponding authors.

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