# Induction of $G_2$/M phase arrest and apoptosis by ZGDHU-1 in A549 and RERF-LC-MA lung cancer cells

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**Abstract.** Lung cancer is a major public health issue worldwide and is associated with high mortality and poor prognosis. Chemotherapy has the potential to reduce tumor size, increase operability and eradicate micrometastases; therefore, novel chemicals to treat lung cancer are urgently required. In the present study, the effects of N, N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4, 5-tetrazine-1,4-dicarboamide (ZGDHu-1), a novel tetrazine derivative, were investigated in A549 and RERF-LC-MA lung cancer cells, and the underlying molecular mechanism of ZGDHu in treating lung cancer was determined. Following incubation with different concentrations of ZGDHu-1, flow cytometry analysis results indicated that ZGDHu-1 could induce $G_2$/mitotic (M) cell cycle arrest and apoptosis in A549 and RERF-LC-MA cells in a dose-dependent manner. Furthermore, western blot analysis demonstrated that the expression levels of $G_2$/M regulatory molecules, including cyclin B1, Cdc2 and cell division cycle 25c, decreased following treatment with ZGDHu-1, whilst p53 expression increased. In addition, A549 and RERF-LC-MA cell apoptosis was induced by cell cycle arrest at the $G_2$/M phase and through the downregulation of nuclear factor-κB. These results suggest that ZGDHu-1 may induce $G_2$/M phase arrest and apoptosis of lung cancer cells, and may serve as a potential therapeutic drug for the treatment of lung cancer.

## Introduction

Cancer is the predominant cause of mortality in the United States and various other countries worldwide (1). Lung cancer is a major health issue worldwide and is primarily caused by tobacco smoking (2-5). Clinically, lung cancer may be categorized into two subtypes: Small cell lung cancer (SCLC) and non-SCLC (NSCLC) (6). For the majority of lung cancer cases, the average survival time from diagnosis is only 8 months (7). In China, lung cancer has been the most common cancer diagnosis and leading cause of cancer-associated mortality for a number of years (8), with previous studies describing an increasing trend (9,10). At present, treatment for lung cancer includes surgery, chemotherapy and radiotherapy. Whilst surgery is considered to be the optimal choice, only 20-25% of lung tumors are suitable for potentially curative resection (11). Two individual participant data meta-analyses reported that postoperative chemotherapy, with or without radiotherapy, improved survival (11). Preoperative chemotherapy has the potential to reduce tumor size, increase operability and eradicate micrometastases. However, chemotherapy may also be ineffective, resulting in delayed surgery with tumors possibly becoming unresectable (11,12). Therefore, exploiting novel chemicals is important to potentially improve the treatment of lung cancer.

N,N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4, 5-tetrazine-1,4-dicarboamide (ZGDHu-1) is a novel tetrazine derivative synthesized by Wei-xiao Hu (Pharmaceutical College of Zhejiang University of Technology, China) who obtained a patent for this chemical in China (13,14). Previous studies have demonstrated that ZGDHu-1 inhibits proliferation, induces apoptosis (15,16) and markedly suppresses the cell cycle at the $G_2$/mitotic (M) phase (17) in leukemia cells. Furthermore, it has been reported that ZGDHu-1 possesses anti-tumor activity, and may induce apoptosis and inhibit proliferation in lung cancer cells (18). However, the mechanisms by which ZGDHu-1 functions to inhibit the cell cycle in human lung cancer cells remain to be elucidated.

The cell cycle is a complex and precise process, and includes M, $G_1$, S and $G_2$ phases. Regulation of the cell cycle predominantly depends on the regulatory network, which includes cyclin-dependent kinases (CDKs), cyclins and cyclin-dependent kinase inhibitors (CKIs) (19,20). $G_2$/M is important for the entrance of cells into M phase, and has also been associated with resistance of tumor cells to chemotherapy (21). During the $G_2$/M arrest, the expression of the Cdc2/cyclin B1 (also known as CDK1) complex is altered,

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resulting in incomplete mitosis and mitotic catastrophe, which induces cell death (17).

The current study aimed to investigate the mechanism by which ZGDHu-1 induces apoptosis and $G_2$/M phase arrest in A549 and RERF-LC-MA lung cancer cells.

## Materials and methods

*Cell culture*. The A549 and RERF-LC-MA human lung cancer cell lines were provided by Dr. Hong Wang (Department of Respiratory Medicine, Zhejiang Provincial People's Hospital, Hangzhou, China). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), HEPES, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere with 5% $CO_2$ at 37˚C.

*Preparation of ZGDHu-1*. ZGDHu-1 was provided by the Pharmaceutical Engineering Research Institute, College of Pharmaceutical Science, Zhejiang University of Technology (Hangzhou, China). ZGDHu-1 was dissolved in dimethyl sulfoxide as a stock solution (1 mg/ml) and stored at -20˚C. For the experiment, the final working concentration (10 $\mu$g/ml) was resuspended in the RPMI 1640 media supplemented with 10% FBS.

*Flow cytometry cell cycle and DNA ploidy analysis*. DNA Prep™ reagent system (Beckman Coulter, Inc., Indianapolis, IN, USA) was used to analyze cell cycle alterations and DNA ploidy in A549 and RERF-LC-MA cells, respectively. Firstly, A549 or RERF-LC-MA cells ($2x10^8$ cells/l) were seeded into 6-well plates overnight and exposed to various concentrations of ZGDHu-1 (2, 10, 50, 100, 200 and 500 $\mu$g/l), RPMI 1640 medium (negative control) or fluorouracil (5-FU; 5 ng/l; positive control; Tianjin Jinyao Amino Acid Co., Ltd., Tianjin, China) for 24 h or 48 h. Cells were subsequently harvested with trypsin, collected by centrifugation (192 x g for 5 min) and washed twice with cold PBS. The pellet was incubated with 50 $\mu$l DNA PREP LPR (containing RNase; Beckman Coulter, Inc.) for 1 min and then treated with DNA PREP stain [containing propidium iodide (PI), Beckman Coulter, Inc.] in a dark place for 5 min at room temperature. Following incubation, the samples were analyzed by flow cytometry (Cytomics FC 500; Beckman Coulter, Inc.) and MultiCycle AV software (Phoenix Flow Systems, San Diego, CA, USA).

*Western blot analysis*. To study the potential molecular mechanism of ZGDHu-1 treatment on A549 and RERF-LC-MA cells, the expression levels of relative proteins was measured by western blot. The A549 and RERF-LC-MA cells were seeded in dishes at a density of $2x10^8$ cells/l, and were cultured overnight. Subsequently, the A549 and RERF-LC-MA cells were treated with different concentrations of ZGDHu-1 (0, 100, 200 and 500 $\mu$g/l) for 48 h. The cells were then collected and lysed using radioimmunoprecipitation assay lysis buffer (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). Protein was extracted and quantified using the BCA Protein Quantitation kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) following the manufacturer's protocol. For each sample, a total of 50 $\mu$g protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10-12%) and transferred onto a polyvinylidene fluoride membrane (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). The membranes were blocked with 10% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST) for 2 h and then incubated with primary antibodies overnight at 4˚C individually. After washing with TBST three times, the membranes were hybridized with a horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h. Detection was performed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). All protein levels were normalized to β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The following antibodies were used: Monoclonal mouse anti-Cdc2 (#9116), monoclonal rabbit anti-human cell division cycle 25c (Cdc25c; #4688), monoclonal mouse anti-cyclin B1 (#4135), polyclonal anti-IκBα (#9242), polyclonal anti-nuclear factor (NF)-κB (#3034) (Cell Signaling Technology, Inc., Danvers, MA, USA), monoclonal mouse anti-p53 (#3036; Biovision, Inc., Milpitas, CA, USA), HRP-conjugated goat anti-mouse immunoglobulin (Ig)G (h+l) and HRP-conjugated goat anti-goat immunoglobulin (Ig)G (h+l) [MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China]. The antibodies were diluted by 1:3,000 in TBST.

Table I. Population of sub-$G_1$ hypodiploid A549 and RERF-LC-MA cells with increasing concentrations of ZGDHu-1.

| Concentration, $\mu$g/l | A549 | RERF-LC-MA |
|---|---|---|
| 2 | 10.4±2.2[a] | 5.2±1.5[a] |
| 10 | 14.2±2.4[a] | 9.2±2.1[a] |
| 50 | 25.5±2.6[a] | 11.4±2.6[a] |
| 100 | 29.2±3.5[a] | 16.2±3.3[a] |
| 200 | 30.9±4.6[a] | 27.9±4.1[a] |
| 500 | 41.3±4.8[a] | 33.2±3.3[a] |
| 5-FU | 25.6±4.3[a] | 62.6±5.2[a] |
| Control | 5.1±0.6 | 3.6±1.5 |

[a]P<0.01 vs. control group (n=3). Data are expressed as the mean ± standard deviation. 5-FU, fluorouracil.

*Statistical analysis*. All statistical calculations were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Results are expressed as the mean ± standard deviation. The differences between treated and control groups were analyzed using t-test. Differences were considered to be statistically significant at values of P<0.05.

## Results

*ZGDHu-1 induces A549 and RERF-LC-MA cell apoptosis through the detection of sub-$G_1$ hypodiploid cells*. An increased population of sub-$G_1$ hypodiploid cells serves as a typical marker of apoptosis (22,23). In the present study, following incubation with various concentrations of ZGDHu-1 (2, 10, 50, 100, 200 and 500 $\mu$g/l), RPMI 1640 medium (negative control)

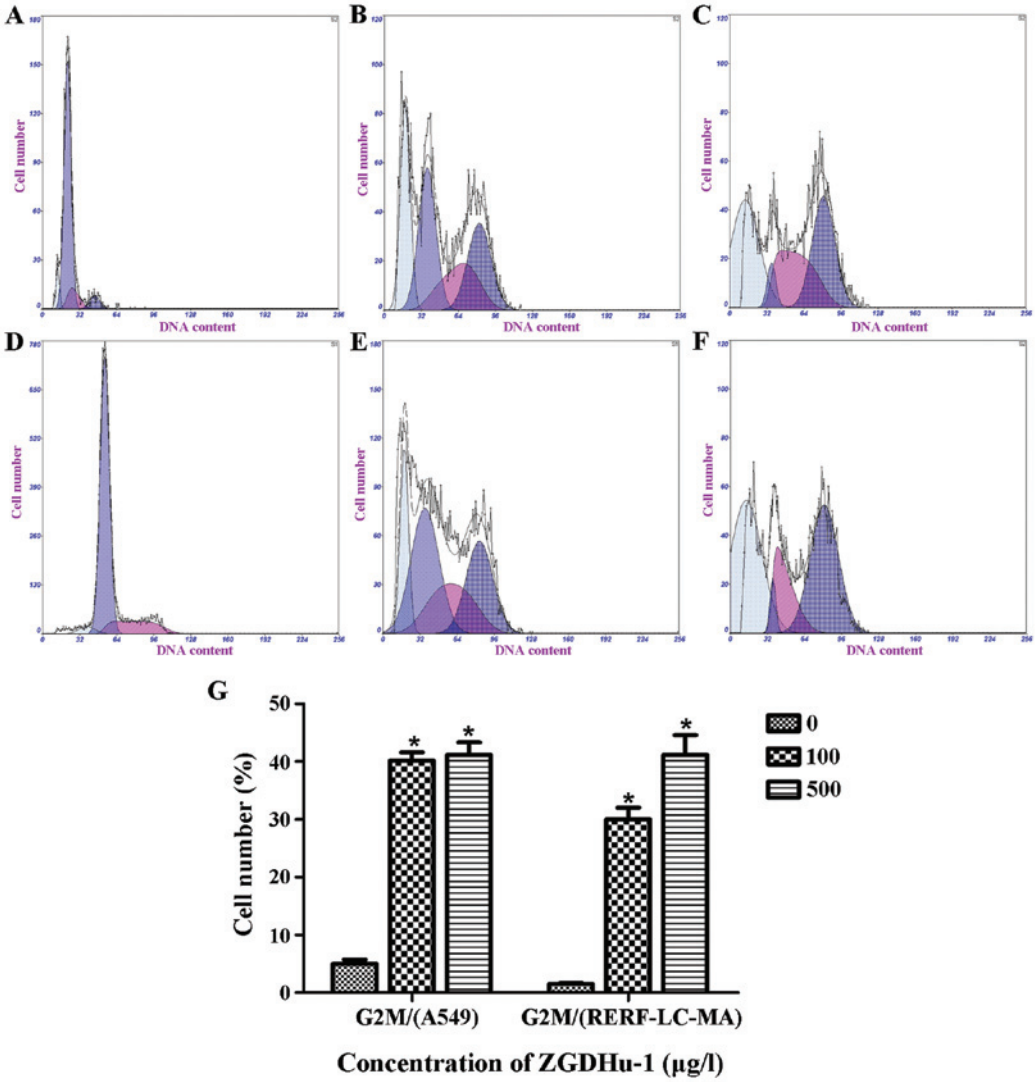


Figure 1. ZGDHu-1 induced $G_2$/M cell cycle arrest in A549 and RERF-LC-MA cells. Cell cycle distribution was analyzed by flow cytometry subsequent to staining with PI. The number of cells in $G_2$/M phase significantly increased with increasing concentrations of ZGDHu-1, and the cell number was 5.02, 44.2 and 41.2% at G2 by (A) 0, (B) 100 and (C) 500$\mu$g/l ZGDHu-1 in A549, respectively. The number of cells in $G_2$/M phase significantly increased with increasing concentrations of ZGDHu-1, and the cell number was 0, 30 and 37.8% at G2 by (D) 0, (E) 100 and (F) 500$\mu$g/l ZGDHu-1 in RERF-LC-MA, respectively. (G) Percentage of cells in $G_2$/M phase in the A549 and RERF-LC-MA cells. Data are expressed as the mean ± standard deviation, and experiments were repeated three times. *P<0.05 vs. control. ZGDHu-1, N, N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4, 5-tetrazine-1,4-dicarboamide; M, mitotic; PI, propidium iodide.

and 5-FU (5 ng/l; positive control) for 48 h, the populations of sub-$G_1$ hypodiploid A549 and RERF-LC-MA cells were analyzed by flow cytometry. The results demonstrated that the sub-$G_1$ hypodiploid cell population increased significantly with increasing concentrations of ZGDHu-1 in the A549 and RERF-LC-MA cells (P<0.01; Table I). In addition, it was observed that the sub-$G_1$ hypodiploid population in the A549 cells was greater than that in the RERF-LC-MA cells. These results suggest that apoptosis is induced by ZGDHu-1, and it may be different in A549 and RERF-LC-MA cells.

*ZGDHu-1 induces cell cycle arrest at the $G_2$/M phase and modulates cell cycle-related protein levels in the A549 and RERF-LC-MA cells*. To determine whether cell cycle changes are involved in ZGDHu-1-induced cell apoptosis, cell cycle phase distribution was detected by flow cytometry. Following treatment of the A549 and RERF-LC-MA cells with various concentrations of ZGDHu-1 (0, 100 and 500 $\mu$g/l) for 48 h, the results indicated that the number of A549 and RERF-LC-MA cells decreased during $G_0/G_1$ phase and increased during $G_2$/M phase with increasing concentrations of ZGDHu-1 (Fig. 1). Furthermore, to investigate the molecular mechanism of ZGDHu-1-induced $G_2$/M arrest in A549 and RERF-LC-MA cells, the expression levels of cell cycle-related proteins, including cyclin B1, Cdc2, Cdc25c and p53, were analyzed by western blotting. The results demonstrated that the protein levels of cyclin B1, Cdc2 and Cdc25c were downregulated in the A549 and RERF-LC-MA cells following treatment with increasing concentrations of ZGDHu-1, whilst the expression of p53 was upregulated (Fig. 2).

*ZGDHu-1 downregulates the expression of NF-κB and upregulates the expression of IκB in A549 and RERF-LC-MA cells*. NF-κB is a nuclear transcription factor, which

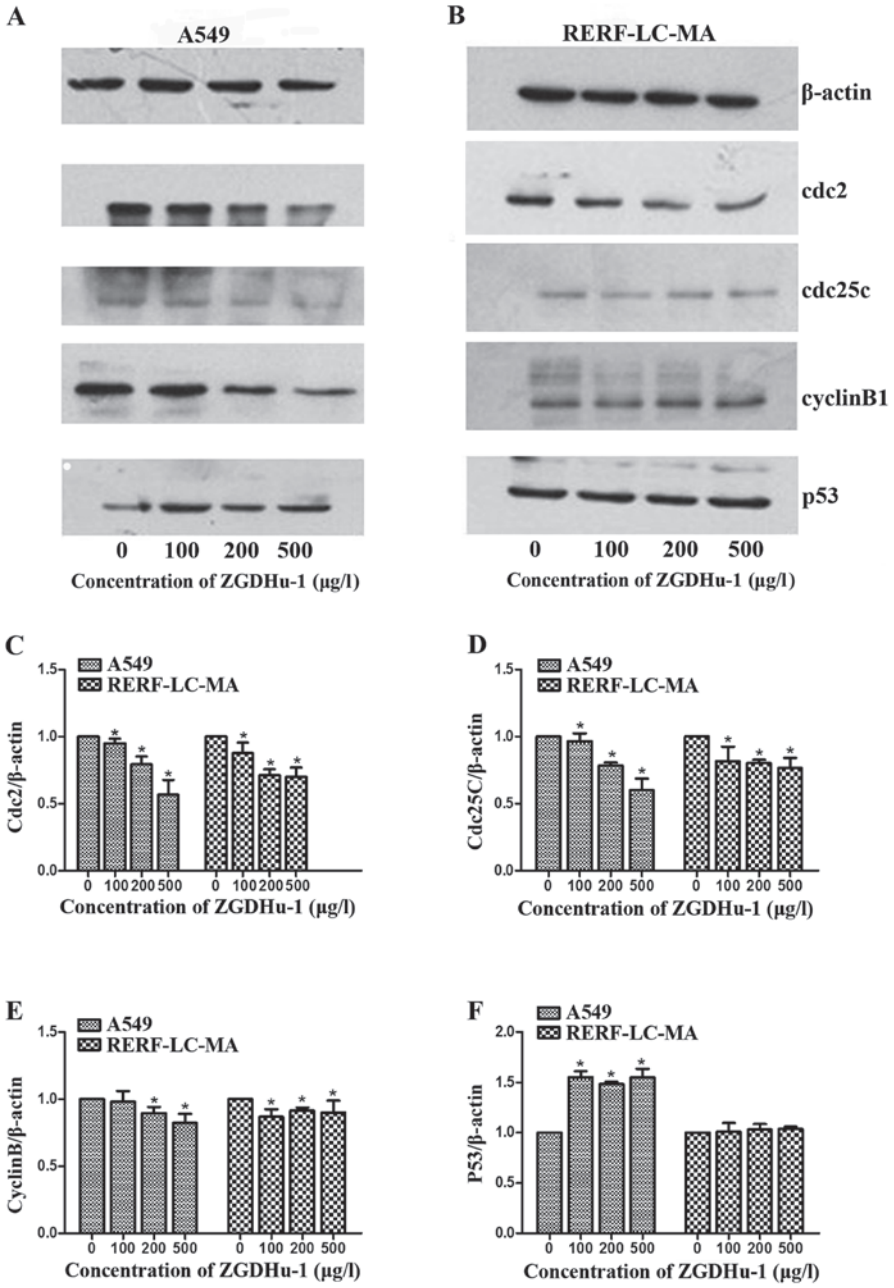


Figure 2. ZGDHu-1 modulates cell cycle-related protein levels in A549 and RERF-LC-MA cells. Western blot analysis of $G_2$/M cell cycle control proteins (cyclin B1, Cdc2, Cdc25c and p53) levels in (A) A549 and (B) RERF-LC-MA cells. Quantification of (C) cdc2, (D) cdc25c,(E) cyclinB1 and (F) P53, respectively. *P<0.05, compared with control The cells were treated with various concentrations of ZGDHU-1 (0, 100, 200 and 500 $\mu$g/l) for 48 h. β-actin was used as a loading control. ZGDHu-1, N, N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4, 5-tetrazine-1,4-dicarboamide; M, mitotic; Cdc25c, cell division cycle 25c.

regulates a number of genes and serves an important role in cellular proliferation, apoptosis, invasion and differentiation. IκB is an inhibitory factor that suppresses the activity of NF-κB (24). In the present study, following incubation with different concentrations of ZGDHu-1 (0, 100, 200 and 500 $\mu$g/l), the expression levels of NF-κB and IκB were detected by western blotting. The results demonstrated that the expression of IκB elevated with the increasing concentrations of ZGDHu-1 in the A549 and RERF-LC-MA cells, whilst the expression of NF-κB decreased (Fig. 3). This suggests that the expression of IκB and NF-κB were altered through the induction of apoptosis in A549 and RERF-LC-MA cells.

## Discussion

Previous studies have reported that ZGDHu-1 is able to inhibit proliferation and induce apoptosis in leukemia cells (15,16), in addition to markedly inhibiting the cell cycle at the $G_2$/M phase (17). Furthermore, it has been demonstrated that ZGDHu-1 inhibits proliferation and induces apoptosis in lung cancer cells (18). However, the mechanisms by which ZGDHu-1 functions to inhibit the cell cycle in human lung cancer cells has not yet been elucidated. In the present study, ZGDHu-1 induced apoptosis through the increased population of sub-$G_1$ hypodiploid cells. In apoptotic cells, DNA is partially degraded, which leaves nucleosomal

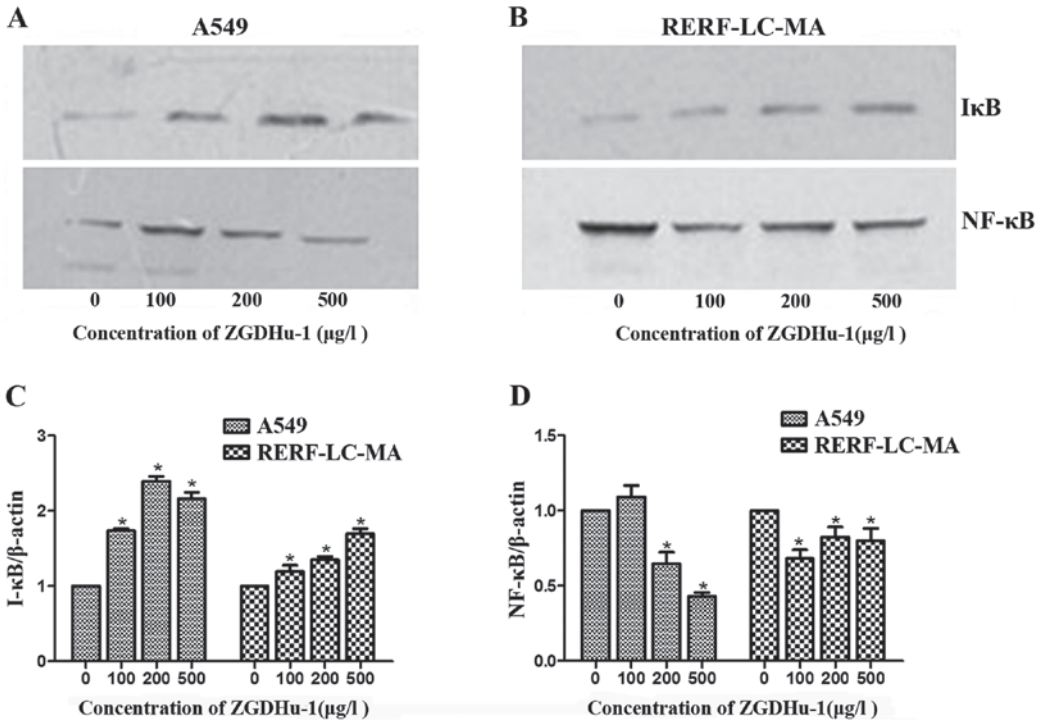


Figure 3. The effect of ZGDHu-1 on the NF-κB pathway in A549 and RERF-LC-MA cells. (A) ZGDHu-1 downregulated the expression of NF-κB and upregulated the expression of IκB in the A549 cells following incubation with various concentrations of ZGDHU-1 (0, 100, 200 and 500 $\mu$g/l) for 48 h. β-actin was used as a loading control. (B) ZGDHu-1 downregulated the expression of NF-κB and upregulated the expression of IκB in the RERF-LC-MA cells following incubation with various concentrations of ZGDHU-1 (0, 100, 200 and 500 $\mu$g/l) for 48 h. Quantification of (C) IκB and (D) NF-κB, respectively. *P<0.05, compared with control. β-actin was used as a loading control. ZGDHu-1, N, N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4, 5-tetrazine-1,4-dicarboamide; NF-κB, nuclear factor-κB.

and oligonucleosomal DNA fragments. PI is a fluorogenic compound. It binds to nucleic acids, meaning that fluorescence emission is proportional to the DNA content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometer, they exhibit a broad hypodiploid (sub-$G_1$) peak, which may be easily discriminated from the narrow peak of cells with normal (diploid) DNA content. Overall, the results of the present study suggested that ZGDHu-1 may induce A549 and RERF-LC-MA cells to undergo apoptosis. Furthermore, we found an interesting phenomenon that the population of sub-$G_1$ hypodiploid were significantly differences in A549 and RERF-LC-MA. Clinically, lung cancer is classified into two subtypes: SCLC (for example, RERF-LC-MA cells) and NSCLC (for example, A549 cells). The SCLC cases are associated with greater chemosensitivity than NSCLC (12,25), thus A549 and RERF-LC-MA cells may exert varying levels of resistance against ZGDHu-1.

The cell cycle may be divided into four stages: $G_1$, S, $G_2$ and M phases. $G_2$/M is important for the entrance of cells into the M phase and is also associated with tumor cell resistance (21). In the present study, it was demonstrated that ZGDHu-1 arrested the cell cycle of the A549 and RERF-LC-MA cells at $G_2$/M phase in a concentration-dependent manner. The regulation of the cell cycle primarily depends on a number of proteins and kinases, which include CDKs, cyclins and CKIs (19,20). The activity of the CDK1 complex is key for the transition from $G_2$ to M in eukaryotic cells (26). Cdc25c is also a CDK, which phosphatase is responsible for dephosphorylating resulting in the activation of the CDK1 complex at the $G_2$/M checkpoint (27). In the present study, expression of cyclin B1, Cdc2 and Cdc25c was downregulated following cell treatment with ZGDHu-1, which suggests that Cdc25c was decreased to inactivate the CDK1 complex, resulting in obstruction of mitotic entry in the A549 and RERF-LC-MA cells. p53, a notable tumor suppressor, is capable of inducing either apoptosis or cell cycle arrest at the cell cycle checkpoints (27,28). Furthermore, p21, a CDK inhibitor, is able to inhibit the CDK-cyclin complexes that are transcriptionally activated by p53 (27). The present study demonstrated that the expression of p53 was upregulated by ZGDHu-1 in a concentration-dependent manner; therefore, p53 was activated and the CDK1 complex was inhibited. These results indicate that apoptosis and $G_2$/M arrest were induced by ZGDHu-1 in the A549 and RERF-LC-MA cells in a concentration-dependent manner.

NF-κB is a heterodimer consisting of two subunits, and is bound to and retained in the cytoplasm by the inhibitor, IκB (29). NF-κB serves a critical role in the promotion of cell growth and proliferation, and the inhibition of apoptosis (30,31). Notably, previous studies have reported that high levels of NF-κB were activated in lung cancer, and inhibition of NF-κB by IκB may suppress lung cancer cell survival and proliferation (32,33). In addition, studies have reported that ZGDHu-1 may upregulate the expression of IκB and downregulate the expression of NF-κB (17). This suggests that IκB levels may have been upregulated by ZGDHu-1 to suppress the function of NF-κB, subsequently inducing apoptosis and $G_2$/M arrest in the A549 and RERF-LC-MA cells in the present study.

In conclusion, the current study demonstrated that ZGDHu-1 is able to induce apoptosis and $G_2$/M arrest in A549 and RERF-LC-MA cells. Notably, cell cycle- and apoptosis-related proteins are key factors that contribute to the inhibitory effects of ZGDHu-1. The present results indicate that ZGDHu-1 may function as a potential, novel drug to treat lung cancer in the future.

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