N,N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide (ZGDHu-1) suppresses the proliferation of PANC-1 pancreatic cancer cells via apoptosis and G2/M cell cycle arrest

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Abstract. Pancreatic cancer is one of the human gastrointestinal malignancies with a high mortality and poor prognosis. Approximately eighty percent of patients are diagnosed with unresectable or metastatic disease. Thus, development of novel chemicals in the treatment of pancreatic cancer is imperative. This study aimed to investigate the anticancer effects of N,N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide (ZGDHu-1), a new tetrazine derivative, on the PANC-1 pancreatic cancer cell line and clarify the underlying molecular mechanism. Using an MTT assay, we found that ZGDHu-1 significantly suppressed the proliferation of PANC-1 cells in a time- and dose-dependent manner. Moreover, according to the morphological and flow cytometric analysis, the results indicated that ZGDHu-1 induced PANC-1 cell apoptosis and G2/M cell cycle arrest in a dose-dependent manner. In the western blot analysis, expression of the pro-apoptotic *Bax* gene was upregulated while the anti-apoptotic *Bcl-2* gene was downregulated following treatment with ZGDHu-1. ZGDHu-1 also activated pro-caspase-3 and PARP and increased the expression of NF-κB inhibitor IκB. Furthermore, the expression levels of G2/M regulatory molecules such as cyclin B1 and cdc2 were decreased while that of Chk1 was increased. These results suggested that ZGDHu-1 suppressed the proliferation of pancreatic cancer cells, rendering it a potential therapeutic drug for the treatment of pancreatic cancer.

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Introduction

Pancreatic cancer, one of the most lethal human gastrointestinal malignancies, is the fourth leading cause of cancer mortality and almost 1/10,000 individuals are diagnosed annually in the United States (1). Pancreatic cancer is characterized by late diagnosis, strong local invasion, early metastasis, high lethal rate, poor prognosis, low long-term survival and <5% of five-year survival rate (2), and is consequently known as 'the king of cancer'.

In China, the incidence of pancreatic cancer ranks 7th among all tumors while it is ranked 6th with regard to mortality rate in all cancer types (3). Improvement of living standards and changes of the diet structure, have led to an upward tendency of the incidence of pancreatic cancer in recent years, particularly in young men (4).

In clinic, 90-95% of the exocrine pancreatic carcinoma is pancreatic ductal adenocarcinoma cancer (5). Studies have shown that the median survival time of pancreatic cancer without surgical resection following diagnosis is 3-6 months, which may be extended to 23 months following surgical resection. However, only 10-15% of diagnosed patients can be completely resected, even if the 5-year survival rate of these patients is only 10% (5,6). In addition, almost all of the patients with pancreatic cancer succumb to the disease within seven years after surgery.

Current methods of treatment for pancreatic cancer include surgery, chemotherapy, radiotherapy and biological therapy, with surgical excision remaining as the only effective and treatable means (7-9). Moreover, low pancreatic resection, high postoperative recurrence and metastasis, and common resistance to chemotherapeutic drugs are the common courses of death in pancreatic cancer patients. Therefore, exploiting novel chemicals is the key to improving the prognosis of pancreatic cancer.

At present, temozolomide, one of the imidazole tetrazines, has already been applied in the clinic and exhibits improved curative effect on leukemia, lymphoma and solid tumors. ZGDHu-1 (N,N'-di-(m-methylphenyi)-3,6-dimethyl-1,4-

dihydro-1,2,4,5-tetrazine-1,4-dicarboamide), which was used in this study is a new tetrazine derivative and was initially designed and produced by W.-X. Hu (Pharmaceutical College of Zhejiang University of Technology, China) (10,11). Moreover,it was previously found that ZGDHu-1 has antitumor activities *in vitro* and *in vivo* and inhibits the proliferation of myeloid leukemia (12) and lung cancer (13), inducing cell apoptosis and differentiation. However, little is known concerning the underlying effect and mechanism of ZGDHu-1 on human pancreatic cancer cells.

In this study, following treatment with this tetrazine derivative, we found that ZGDHu-1 inhibited the proliferative effect and enhanced apoptosis in PANC-1 cells, and blocked its cell cycle at G2/M phase. Furthermore, the mitochondrial pathway and activation of caspase-3 are important in ZGDHu-1-induced apoptosis, and the nuclear factor inhibitor IκB was also involved in this process. CyclinB1, cdc2 and Chk1 are G2/M regulatory molecules, whose expression levels were altered following the treatment of ZGDHu-1.

Materials and methods

Cell culture and reagents. The human PANC-1 pancreatic cancer cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 0.25% Trypsin-EDTA were purchased from Dingguo Biotechnology Co., Ltd. (Gibco, Grand Island, NY, USA). Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2–yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst-33258 apoptosis kit (no. B1155) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The DNA prep kit (no. 6607055) was purchased from Beckman Coulter, Inc. (Brea, CA, USA). The Annexin V/Dead Cell Apoptosis kit (no. V13241) was purchased from MultiSciences Biotech Co., Ltd. (Invitrogen, Carlsbad, CA, USA), and the ECL Western Blotting Substrate kit (no. P1425) was purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). The RIPA lysis buffer (no. WB-0071) and BCA Protein Quantitation kit (no. BCA02) were purchased from DingGuo Biotechnology Co., Ltd. (Beijing, China), and PageRuler™ Prestained Protein Marker (no. 26616) was purchased from Thermo Scientific (Rockford, IL, USA). Primary antibodies against Caspase-3 (no. 9665), cleaved-Caspase-3 (no. 9661), PARP (no. 9532), Bax (no. 2774), NF-κB (no. 3034), IκB (no. 9242), Chk1 (no. 2345), cyclinB1 (no. 4135), cdc2 (no. 9116) and α-tubulin (no. 3873) were purchased from Cell Signaling Technology (Danvers, MA, USA), and Bcl-2(sc-7382) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against β-actin and secondary antibodies were purchased from MultiSciences Biotech Co., Ltd. Drug solutions were freshly prepared on the day of testing.

Preparation of ZGDHu-1. ZGDHu-1 was kindly provided by the Pharmaceutical Engineering Research Institute, College of Pharmaceutical Science, Zhejiang University of Technology. ZGDHu-1 was dissolved in DMSO (maximum concentration, 10 mg/ml) and stored at -20˚C for use. Prior to the *in vitro* experiment, DMEM was used to dilute the stock solution (10 mg/ml) to the final working concentration.

Cell viability assay. The MTT assay was used to measure the cell viability of PANC-1 cells. Briefly, PANC-1 cells were seeded in 96-well plates at a density of $8x10^3$ cells per well and incubated overnight at 37˚C in 10% FBS medium. The cells were then treated with increasing concentrations (0, 0.01, 0.05, 0.1, 0.2, 0.5 and 1 μg/ml of ZGDHu-1 or DMEM medium (control). After incubation for 24, 48 and 72 h at 37˚C, 20 μl of MTT solution (5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37˚C, and 150 μl of DMSO was added to dissolve the crystal. The optical density (OD) value at 490 nm was determined using a microplate reader (Rayto, USA). Cell viability was calculated as: Cell viability (%) = $[(OD_{sample}-OD_{blank})/(OD_{control}-OD_{blank})]$ x100.

Morphological analysis. To evaluate the apoptotic activity of PANC-1 cells, we used Hoechst-33258 and Wright's staining to observe the changes of the cell nucleus. Cover glasses were initially placed in 6-well plates. The PANC-1 cells ($5x10^5$ cells in 2 ml) were subsequently seeded in 6-well plates overnight and exposed to increasing concentrations of ZGDHu-1 or DMEM medium (control) for 24 and 48 h. The cells for Hoechst-33258 were fixed with fixative (methanol:glacial acetic acid 3:1) for 5 min at 4˚C and incubated with Hoechst-33258 (5 μg/ml) for 15 min after washing with PBS. The cells were then observed under a fluorescence microscope (Nikon Y-THS, Japan). However, the cells for Wright's staining were stained with Wright A and B for 10 min and observed under a light microscope (Olympus CHA, Japan).

Detection of cell apoptosis. The Annexin V/Dead Cell Apoptosis kit was used to detect PANC-1 cell apoptosis by flow cytometry. Firstly, PANC-1 cells ($5x10^5$ cells in 2 ml) were seeded in 6-well plates overnight and exposed to increasing concentrations of ZGDHu-1 or DMEM medium (control) for 24 h. The cells were harvested with trypsin, collected by centrifugation (192 x g for 5 min), and washed twice with cold PBS. Cells at $5x10^5$ cells/ml were resuspended in 1X Annexin V binding buffer at a final concentration of $1x10^6$ cells/ml. The cells were stained with 5 μl of Annexin V-FITC and 1 μl of PI (100 μg/ml) for 15 min at room temperature in the dark. After incubation, 400 μl of 1X Annexin V binding buffer were added to an adequate cell dilution and detected by flow cytometry (FC500, Beckman Coulter, Inc.) using 488 nm excitation.

Flow cytometry cell cycle analysis. PANC-1 cells were treated as described above. Briefly, after harvesting, centrifugation (192 x g for 5 min) and washing, the pellet was incubated with 50 μl of DNA PREP LPR (containing RNase) for 1 min and then treated with DNA PREP™ Stain (containing PI) in the dark for at least 5 min. The samples were analyzed by flow cytometry (FC500, Beckman Coulter, Inc.) and at least 10,000 cells were counted.

Western blot analysis. To identify the potential molecular mechanism of ZGDHu-1 on PANC-1 cells, expression levels of relative proteins were measured by western blotting.

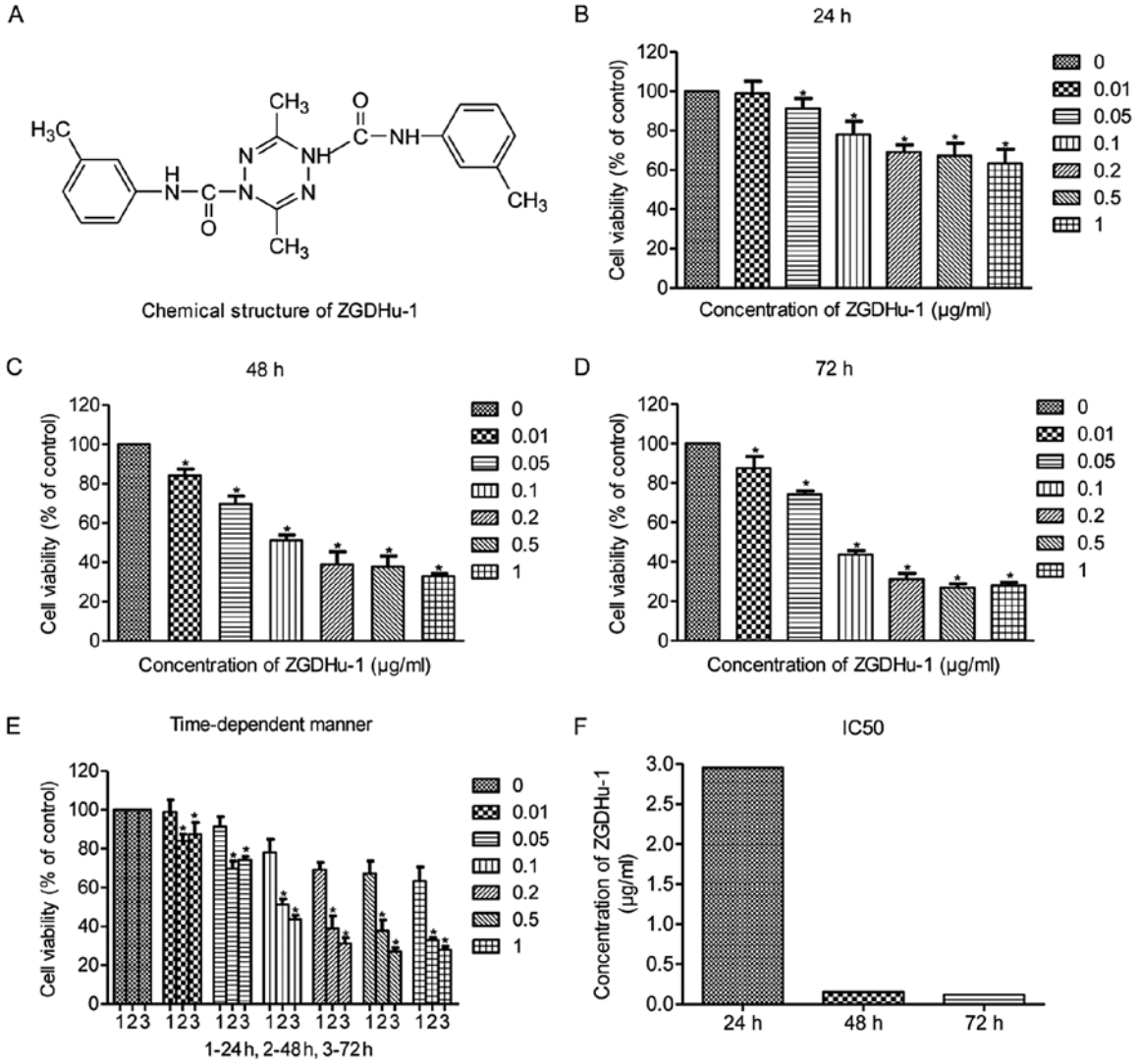

Figure 1. ZGDHu-1 suppresses the proliferation of PANC-1 cells. (A) Chemical structure of ZGDHu-1. (B-F) PANC-1 cells were cultured with various concentrations of ZGDHu-1 (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1 μg/ml) for 24, 48 and 72 h and detected by MTT assay. Data was shown as means ± standard and at least three representative experiments were performed. $^{*}p<0.05$, compared with control.

PANC-1 cells were seeded in the 75 cm^2 dishes at a density of $2.5x10^6$ cells/dish. After being cultured overnight, PANC-1 cells were treated with different concentrations of ZGDHu-1 for 24 and 48 h, respectively. PANC-1 cells were then collected and lysed using RIPA lysis buffer and the protein concentration was determined using a BCA protein quantification kit following the manufacturer's instructions. For each sample, an equal amount of protein (50 μg) was separated by SDS-PAGE (10-12%) and transferred onto a PVDF membrane at the constant current of 200 mA for 60 min. The membrane was blocked with 10% non-fat dried milk in Tris-buffered saline with Tween-20 (TBST) for 2 h and then incubated with primary antibodies (1:1,000) overnight at 4˚C individually. After washing with TBST three times, the membranes were hybridized with secondary antibody-conjugated horseradish peroxidase (goat anti-mouse 1:10,000, goat anti-rabbit 1:10,000) at room temperature for 2 h. The ECL Western blotting substrate kit was added on the membranes for reaction and the fluorescence of the protein bands was exposed to X-ray film. All the protein levels were normalized by β-actin or α-tubulin.

Statistical analysis. Results are presented as the means ± standard deviation. The significance of differences between two groups was determined using the t-test. All the results were obtained from three separate experiments. Differences were considered to be statistically significant at values of $p<0.05$.

Results

ZGDHu-1 suppresses the proliferation of PANC-1 cells. The structure of ZGDHu-1, a new tetrazine derivative, is shown in Fig. 1A. We firstly evaluated the cytotoxic efficacy of ZGDHu-1 on the PANC-1 pancreatic cancer cell line by using MTT assay. Following treatment with various concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1 or 5 μg/ml) of ZGDHu-1 for 24, 48 and 72 h, the results demonstrated that ZGDHu-1 inhibited the proliferation of PANC-1 cells in a time- and dose-dependent manner (Fig. 1B-E). Moreover, the half maximal inhibitory concentration (IC_{50}) of 24, 48 and 72 h for PANC-1 cells was 2.95, 0.15 and 0.12 μg/ml of ZGDHu-1 (Fig. 1F). Therefore, concentrations of 0.1, 0.2 and 0.5 μg/ml of ZGDHu-1 on the cells were used for the subsequent experiments.

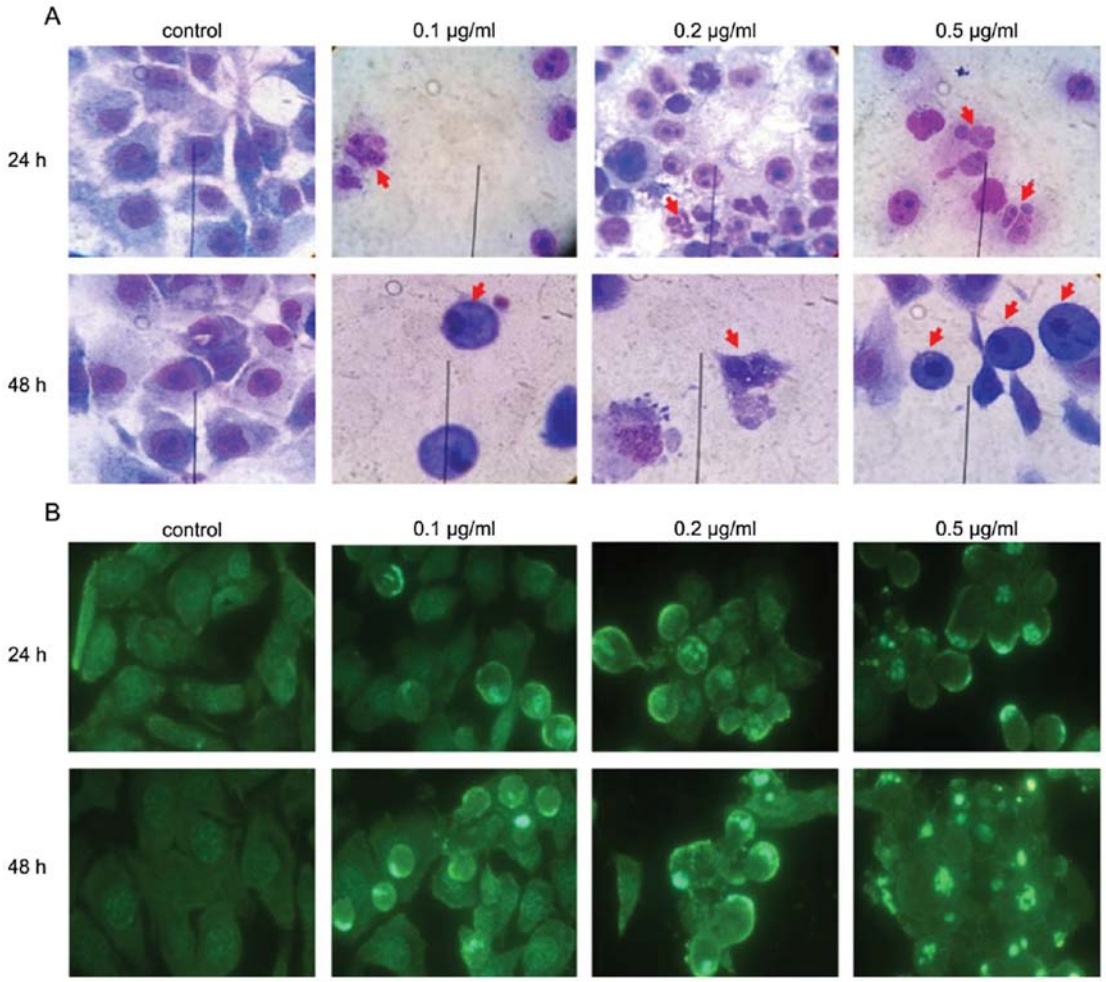

Figure 2. The morphology observation of ZGDHu-1 treatment on PANC-1 cells. PANC-1 cells cultured with 0, 0.1, 0.2 and 0.5 μg/ml of ZGDHu-1 for 24 and 48 h. (A) PANC-1 cells were dyed with Wright's staining and observed under a light microscope (x1,000). (B) PANC-1 cells were dyed with Hoechst-33258 staining and observed under a fluorescence microscope (x400).

ZGDHu-1 induces apoptosis on PANC-1 cells. To confirm whether the ZGDHu-1-induced inhibition of PANC-1 cell proliferation was caused by apoptosis, PANC-1 cells treated with ZGDHu-1 were observed under a microscope. After the treatment of ZGDHu-1, the morphologic features of apoptosis were significantly changed, such as cell shrinkage, nuclear chromatin condensation and fragmentation as well as the formation of apoptotic bodies, particularly evident in higher concentrations of ZGDHu-1 (Fig. 2A and B). Additionally, when using the Annexin V/Dead Cell Apoptosis kit to detect the early apoptotic effect of ZGDHu-1 on PANC-1 cells, we found that the apoptotic effect of ZGDHu-1 on PANC-1 cells occurred in a dose-dependent manner. In addition, the early apoptotic rates of PANC-1 cells were 3.6, 12.6, 17.1 and 18.5%, while the total apoptotic rates were 4.5, 14.2, 18.6 and 20.8% at concentrations of 0, 0.1, 0.2 and 0.5 μg/ml of ZGDHu-1 (Fig. 3). Thus, these findings indicated that ZGDHu-1 can induce apoptosis of PANC-1 cells and the apoptotic rates of PANC-1 cells were elevated with the increasing concentration of ZGDHu-1 treatment.

ZGDHu-1 upregulates the expression level of IκB. NF-κB is a vital nuclear transcription factor that regulates many genes and participates in cell proliferation, apoptosis, invasion and differentiation (14). In this study, we observed that the expression of NF-κB was not significantly changed while its inhibitor IκB was strongly increased with the addition of ZGDHu-1 in a dose-dependent manner especially after treatment with ZGDHu-1 for 48 h (Fig. 4).

ZGDHu-1 induces cell cycle arrest at G2/M phase and modulates cell cycle-related protein levels in PANC-1 cells. To determine whether cell-cycle changes are involved in ZGDHu-1-induced cell apoptosis, we detected cell-cycle phase distribution by flow cytometry. Following the treatment of PANC-1 cells with different concentrations of ZGDHu-1 (0, 0.1, 0.2 and 0.5 μg/ml) for 24 and 48 h, we found that the PANC-1 cells decreased at G0/G1 phase and increased at G2/M phase with the increasing concentrations of ZGDHu-1 (Fig. 5). The percentages of cells at G2/M phase were 9.6, 18.25, 27.7 and 73.01% at 24 h and 17.9, 23.7, 30.9 and 48.62% at 48 h, respectively. Furthermore, to investigate the molecular mechanism for ZGDHu-1-induced G2/M arrest in PANC-1 cells, we detected the expression levels of cell cycle-related proteins such as Chk1, cyclinB1 and cdc2. The results showed that the protein levels of cyclinB1 and cdc2 were downregulated, while the expression of Chk1 was increased following treatment with ZGDHu-1, especially when treated at 48 h (Fig. 6).

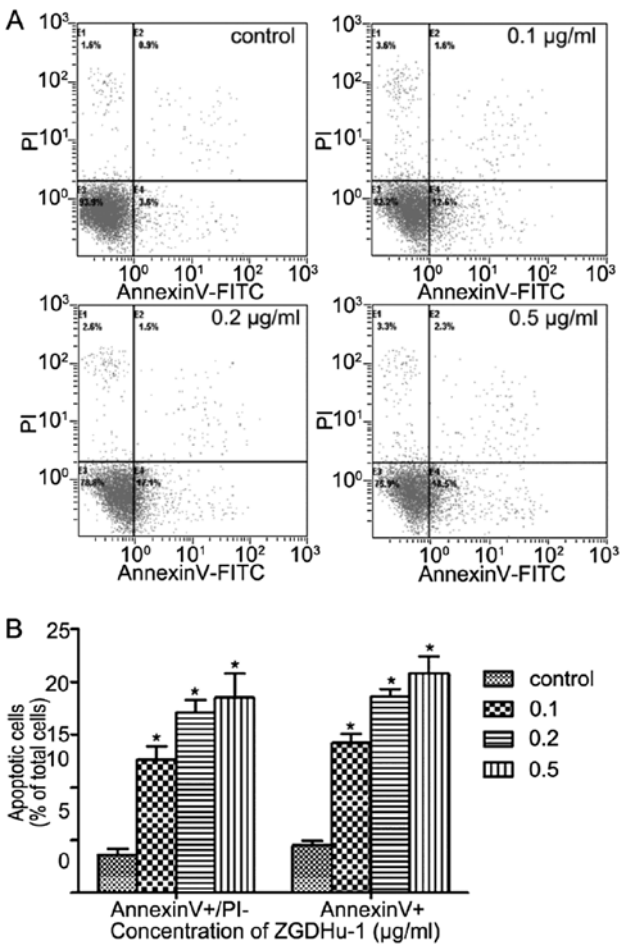

Figure 3. Apoptosis analysis of PANC-1 cells with the treatment of ZGDHu-1. ZGDHu-1 induced apoptosis in PANC-1 cells. (A) PANC-1 cells were treated with 0, 0.1, 0.2 and 0.5 μg/ml of ZGDHu-1 for 24 h and the apoptotic rate was determined by flow cytometry with Annexin V-FITC/PI double dye assay. (B) Annexin V^+/PI^- represented early apoptosis and Annexin V^+ represented total apoptosis (include early and late apoptosis) as shown in the histogram. Data are presented as the means ± standard deviation. Experiments were repeated three times. $^*P<0.05$, compared with the control.

ZGDHu-1-induced PANC-1 cell apoptosis via mitochondrial apoptosis pathway and the activation of caspase-3. The mitochondrial pathway is important in the apoptotic process. To examine whether ZGDHu-1 induced apoptosis of PANC-1 cells through this pathway, we analyzed the expression of Bcl-2 and Bax protein after treatment with ZGDHu-1 at concentrations of 0, 0.1, 0.2 and 0.5 μg/ml for 24 and 48 h. The result indicated that the expression of Bax was increased in a dose-dependent manner, whereas Bcl-2 expression was decreased (Fig. 7A). In addition, we found that the expression of pro-caspase-3, PARP (the substrate of caspase-3) was downregulated while cleaved-caspase-3 was significantly upregulated after treatment with ZGDHu-1 (Fig. 7B). These results suggest that ZDGHu-1- induced cell apoptosis occurred through a mitochondrial pathway and the activation of caspase-3.

Discussion

Pancreatic cancer is an aggressive malignant disease due to the lack of early diagnosis and treatment options (15). Moreover,

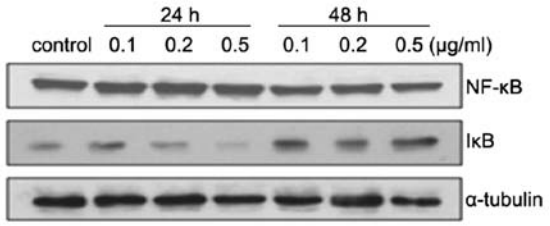

Figure 4. ZGDHu-1 activated the NF-κB pathway in PANC-1 cells. PANC-1 cells were treated with increasing concentrations (0, 0.1, 0.2 and 0.5 μg/ml) for 24 and 48 h. The expression of NF-κB and IκB proteins was detected by western blot assay, and ZGDHu-1 led to the upregulation of IκB.

pancreatic cancer is one of the most intrinsic chemical resistance tumors. Common intrinsic or acquired chemical resistance become the main causes of failure for pancreatic cancer therapy (16). Identification of a new drug that is highly sensitive and has fewer side effects may lead to effective treatments for pancreatic cancer.

ZGDHu-1 has been proven to repress the proliferation of myeloid leukemia (12) and lung cancer (13). Pro-apoptotic effects were demonstrated on those carcinoma cells and were independent of its anti-proliferative action. In this study, we found that ZGDHu-1 inhibited the proliferation of PANC-1 cells in a time- and dose-dependent manner with the treatment of ZGDHu-1 for 24, 48 and 72 h. The apoptotic effects of ZGDHu-1 on PANC-1 cells were enhanced based on the cytomorphology observation and Annexin V/PI staining. Overall, our study has demonstrated that ZGDHu-1 effectively suppressed cell viability and induced apoptosis of PANC-1 cells.

The molecular mechanism of ZGDHu-1 regarding which pathway to induce apoptosis in cancer cells was widely studied. For example, it was demonstrated that the NF-κB pathway may play a key role in ZGDHu-1-induced apoptosis in myeloid leukemia (17), while the results of another study indicated that ZGDHu-1 inhibited lung cancer cells by upregulating the expression levels of pro-apoptotic proteins, such as Bax and p53 (13). Although ZGDHu-1 may induce cell-cycle arrest in cancer cells, the antitumor effects of ZGDHu-1 on pancreatic cancer as well as the associated molecular mechanisms have yet to be elucidated. We have shown that ZGDHu-1 can induce PANC-1 cell apoptosis and G2/M phase arrest.

NF-κB is composed of two subunits and is normally sequestered in the cytoplasm by its inhibitor proteins, IκB (18). When the cells are exposed to NF-κB-activating signals, it can induce the phosphorylation of IκB, targeting them for rapid degradation through a ubiquitin-proteosome pathway, releasing NF-κB to the nucleus, where it binds to specific sequences in the promoter regions of genes (19). Our results have shown that ZGDHu-1 mainly inhibited the growth of PANC-1 cells by upregulating the expression level of IκB, whereas the expression of NF-κB did not change significantly following treatment of ZGDHu-1 because of its higher expression in the cytoplasm.

The cell cycle is an intricate process to ensure the replication of cells that can be divided into four stages: G1 phase, S phase (DNA synthesis), G2 phase, and M phase (Mitosis). In this study, we found that ZGDHu-1 arrested the cell cycle of PANC-1 cells at G2/M phase by using flow cytometry. Previous findings show that the cell-cycle progression

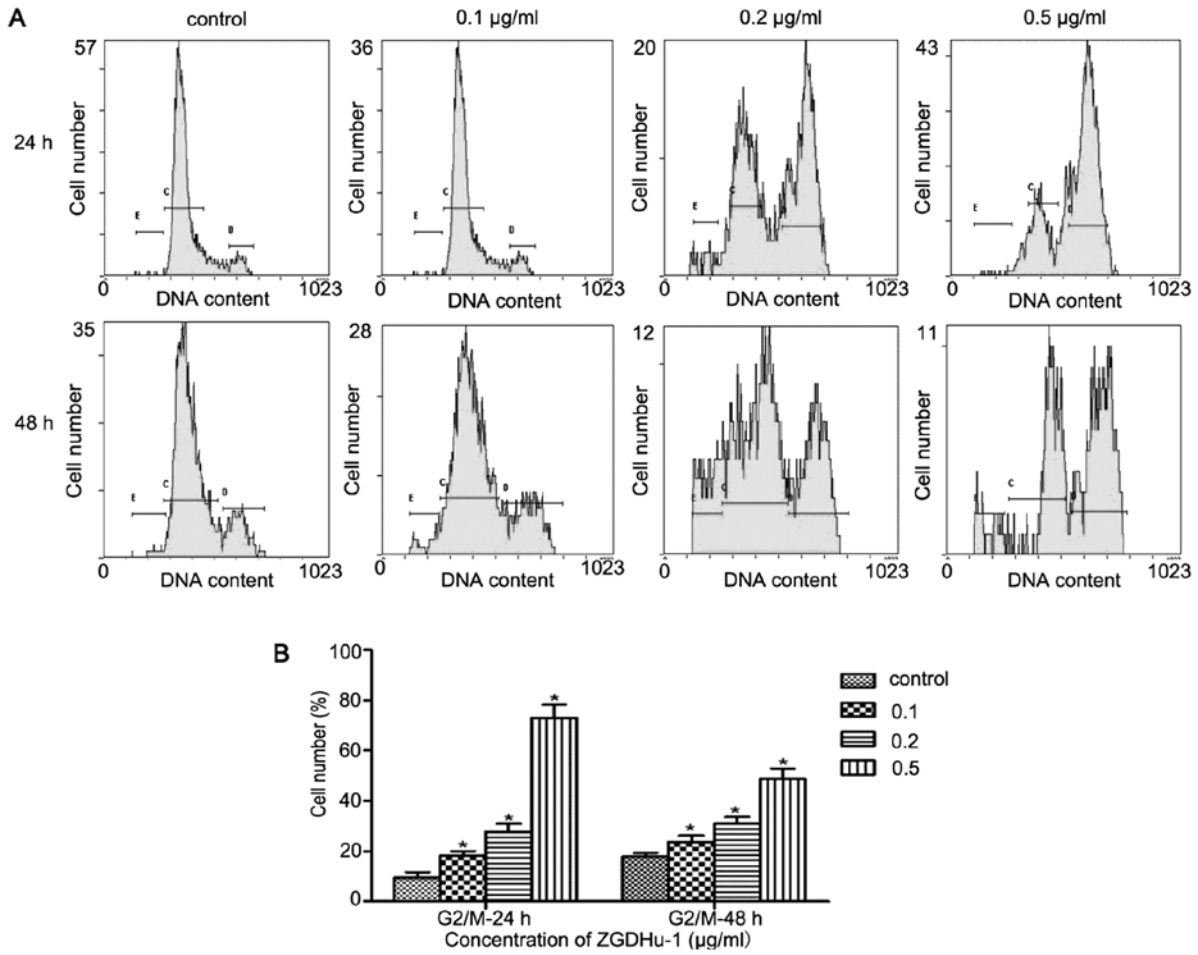

Figure 5. ZGDHu-1 induced G2/M cell cycle arrest in PANC-1 cells. PANC-1 cells were exposed to different concentrations of ZGDHu-1 (0, 0.1, 0.2 and 0.5 μg/ml) for 24 and 48 h. (A) The cell cycle distribution was analyzed by flow cytometry after staining with PI and G2/M was significantly increased. (B) The percentage of cells in G2/M phase was represented by a column diagram. Data are presented as the means ± standard deviation. Experiments were repeated three times. *P<0.05, compared with the control.

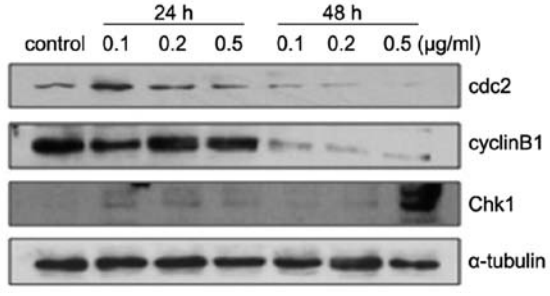

Figure 6. ZGDHu-1 modulated cell cycle-related protein levels in PANC-1 cells. PANC-1 cells cultured with increasing concentrations (0, 0.1, 0.2 and 0.5 μg/ml) for 24 and 48 h. The expression levels of Chk1, cyclinB1 and cdc2 protein were detected by western blot assay.

depends on highly ordered events controlled by cyclins and cyclin-dependent kinases (Cdks). For example, the expression of p21 induced by p53 exerts an important role in the G1/S checkpoint by inactivating Cdk2 in response to DNA damage. However, the transition from G2 to Mitosis principally depends on the activity of the cyclin B1/cdc2 (Cdk1) complex (20). Chk1, a serine/threonine kinase, has been proven an important checkpoint kinase in the cycle arrest through phosphorylating

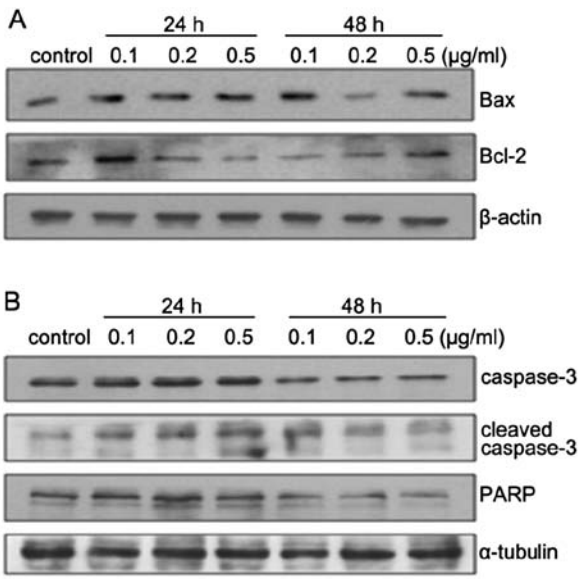

Figure 7. ZGDHu-1-induced PANC-1 cell apoptosis via the upregulation of Bax/Bcl-2 and the activation of caspase-3. PANC-1 cells cultured with escalating dose of ZGDHu-1 (0, 0.1, 0.2 and 0.5 μg/ml) for 24 and 48 h. (A) The expression levels of Bax and Bcl-2 protein were detected by western blot assay. (B) Caspase pathway was activated by assessing the expression levels of caspase-3, cleaved-caspase-3 and PARP using western blot analysis.

cdc25C on Ser-216 (21). Our study has demonstrated that the protein levels of cdc2 and cyclinB1 were obviously decreased in a dose- and time-dependent manner, which blocked the cell cycle entering into mitosis and induce the apoptosis of PANC-1 cells.

There are two main apoptotic pathways that have been extensively investigated: the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways (22). The intrinsic pathway is activated by a myriad of stress signals, such as DNA damage, leading to the mitochondrial outer membrane permeabilization and the release of apoptotic proteins, such as cytochrome *c* and second mitochondrial-derived activator of caspases from mitochondria into cytoplasm. The intrinsic pathway, leads to the release of cytochrome *c*, which is suppressed by Bcl-2 and promoted by Bad (23,24). However, the extrinsic pathway is activated by the binding of death ligands to the cell surface receptors (TNF receptor superfamily), leading to a complex signal transduction into the cytoplasm and activating the initiator caspase-8 and the subsequent executioner caspases.

Caspase-3, the executioner caspase, is the downstream activator of extrinsic and intrinsic apoptotic pathways (25). Furthermore, it is well known that antitumor agents can target caspases and their substrates such as PARP-1 in apoptotic pathways in cancer cells, while the PARP-1-mediated pathway is a major mechanism for DNA repair in cancer cells (26). According to the results of western blotting, we found that the expression of Bax was increased, whereas Bcl-2 was decreased in PANC-1 cells treated with ZGDHu-1 compared with the control group in the present study. In addition, for the expression levels of pro-caspase-3, PARP was downregulated while the cleaved-caspase-3 was greatly upregulated with the increasing concentrations of ZGDHu-1. Our results suggest that ZDGHu-1 induced cell apoptosis through the mitochondrial pathway.

In conclusion, the present study has demonstrated that ZGDHu-1 elaborates the cytotoxic effects against PANC-1 cells by inducing apoptosis and arresting the cell cycle at G2/M phase. The upregulation of pro-apoptotic proteins and activation of caspase-3 are the key factors that contribute to the inhibitory effects of ZGDHu-1. In the future, more and more advancing studies about the effect of ZGDHu-1 in pancreatic cancer will be confirmed, which may be useful with the development of ZGDHu-1 as a new drug for cancer treatment.

Acknowledgements

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