Ursolic acid inhibits growth and induces apoptosis in gemcitabine-resistant human pancreatic cancer via the JNK and PI3K/Akt/NF-κB pathways

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Abstract. Pancreatic cancer is one of the most deadly carcinomas worldwide. Although gemcitabine as the standard chemotherapy agent has been proven to be effective, the response rate remains at 5.4% and the 5-year survival rate is extremely poor. Ursolic acid (UA) is a small molecule compound extracted from Chinese herbs as well as edible vegetables and a well-known anti-inflammatory and immunosuppressive agent. Here, we show that UA has potential to be developed into an anti-neoplastic agent against gemcitabine-resistant pancreatic cancer and to explore its molecular mechanism of action. *In vitro*, we used three different malignancy grades of pancreatic resistant cancer cell lines including MIA PaCa-2, PANC-1 and Capan-1 to assess the antitumor effect of UA. We found that UA inhibited growth and induced apoptosis in a dose-dependent manner in all of the three pancreatic cancer cell lines. Both extrinsic and intrinsic pathways were found to be involved in apoptotic cascade. The potential signaling pathways are concerned with inactivation of the PI3K/Akt/NF-κB pathway and activation of the c-Jun-terminal kinase (JNK) pathway. The JNK inhibitor SP600125 partly abrogated the caspase-9 activation caused by UA. The Akt inhibitor LY294002 did not mimic the effect of UA on caspase-8 and -9, but inhibited the viability of MIA PaCa-2 cells to some extent. Equally, UA also overcame the chemoresistance in the chemoresistant endometrial and ovarian carcinoma cell lines (HEC-1A and OVCAR-3). Moreover, UA caused cytotoxicity to a nude mouse xenograft model *in vivo*. Therefore, our present data suggest that UA can act as a novel and potent therapeutic agent in gemcitabine-resistant pancreatic cancer and even as a promising candidate in other chemoresistant cancers.

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Introduction

Pancreatic cancer, characterized by aggressive malignancy and high resistance to chemotherapy agents, is considered as one of the most lethal tumors with a 5-year mortality of 97-98% (1,2). Surgery is the only curative treatment for pancreatic cancer. However, <20% patients have resectable tumor at the time of diagnosis due to the poor diagnosis (3). Except for the surgical therapy, both of chemotherapy and radiation regimens hardly ameliorate the outcome of pancreatic cancer patients, which causes ~90% of cases death within 1 year after diagnosis (4). Since the end of 20th century, gemcitabine, a deoxycitidine analogue, was confirmed to improve the objective response rate in patients with advanced pancreatic carcinoma, and has been applied as a first-line chemotherapy agent (5,6). However, only 5.4% of all patients are sensitive to the gemcitabine-based chemotherapy (7,8). Thus, novel chemotherapeutic drugs and candidate compounds are urgently needed to improve the outcome of patients with the lethal malignancy.

Ursolic acid (UA) is a pentacyclic triterpenoid compound extracted and purified from many types of medicinal plants, herbs, and edible vegetables (9). UA contains a variety of pharmacological activities. The anti-oxidative and anti-inflammatory actions of UA are widely applied to hepatoprotection, neuroprotection, cardiovascular protection and so on (10-14). Besides these effects, UA has been proved to possess multiple anti-tumor activities including inhibiting tumorigenesis and progression (9). UA causes cytotoxicity to diverse malignancies such as fibrosarcoma, prostate cancer, hepatocellular carcinoma, colorectal cancer, melanoma, and breast cancer (15-20). However, the cytotoxic role of UA in gemcitabine-resistant human pancreatic cancer and its potential molecular mechanisms are still unclear.

In this study, we investigated the effect of UA on growth restriction and apoptosis in human pancreatic resistant cancer *in vitro* and *in vivo*. Additionally, we explored possible mechanisms of action, such as PI3K/Akt/NF-κB and c-Jun N-terminal kinase (JNK) pathways.

Materials and methods

Cell culture and reagents. The human pancreatic resistant cancer cell lines MIA PaCa-2 and PANC-1 were kindly provided by Shanghai Institute of Materia Medica. The human pancreatic

resistant cancer cell line Capan-1 was kindly provided by Department of Pharmacology, Sun Yat-Sen University. The paclitaxel-resistant endometrial cell line HEC-1A was purchased from Cell Bank of Chinese Academy of Sciences. The platinum-resistant ovarian cancer cell line OVCAR-3 was purchased from Experimental Animal Center of Sun Yat-Sen University. The cells were routinely cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO_2 at 37˚C. Ursolic acid (UA) (National Institutes for Food and Drug Control, China) was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) as a 100 mM stock solution and stored at -20˚C. Further dilution was done in serum-free cell culture medium. PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA) was dissolved in DMSO (Sigma) as a 50 mM stock solution and stored at -20˚C. Similarly, JNK inhibitor SP600125 (Sigma) was dissolved and stored as a 10 mM stock solution by DMSO at -20˚C.

Cell viability and proliferation. Cells were seeded into 96-well plates at a density of $2x10^3$/well and allowed to attach overnight before being treated by different concentrations of UA for 24 h. Then cell viability and proliferation were evaluated. Cell viability was tested by the 3-(4,5-dimethylthiaziazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) assay as described in the literature (21). Detection of cell proliferation was through the colorimetric 5'-bromo-2'-deoxy-uridine (BrdU) incorporation assay (Roche Diagnostic Corp., Indianapolis, IN, USA) according to the instruction of the manufacturer. Briefly, cells were labeled by addition of BrdU for 4 h in 5% CO_2 at 37˚C. Then, FixDenat solution was used for cells fixation. The anti-BrdU-POD antibody was added to locate the BrdU label in the DNA. After the incubation of the peroxidase substrate, the immune complex was detected by a multi-well spectrophotometer reader using an iMark™ Microplate Reader (Bio-Rad, Richmond, CA).

Hoechst 33258 staining. The three types of cells at the density of $2x10^6$/well in a 6-well plate were fixed by 4% paraform for 10 min. Then Hoechst 33258 (Sigma) at 5 μg/ml was added to the cells for 15 min at room temperature in the dark. The nuclei morphology was observed by fluorescence microscopy (Olympus, Melvie, NY) with a 340-nm excitation filter.

Caspase-3/7, -8 and -9 activity measurements. Caspase-3/7, -8 and -9 activity were assessed by Caspase-Glo luminescent-based assays (Promega, Madison, WI). Accordance with the instructions, cells ($1x10^4$/well) were seeded into a 96-well plate overnight to ensure adherence. After various concentrations of UA treatment for 24 h in a 5% CO_2-humidified atmosphere at 37˚C, 100 μl of 3/7, -8 and -9 Caspase-Glo reagents were added to each well, respectively. Mixed and incubated for 1 h, the mixtures were transferred to a fluorescence microtiter plate and quantified fluorescence intensity by a luminometer. The corresponding OD values from MTT assay described above were considered as the viable cell number to normalize the fluorescence intensity.

Cytochrome C ELISA assay. Cytoplasmic cytochrome C levels were determined by a cytochrome C ELISA kit (R&D Systems, Minneapolis, MN). The cytoplasmic protein was collected by a nuclear and cytoplasmic extraction reagents (Pierce, Rockford,

IL) from adherent cells ($2x10^6$/well) in a 6-well plate after UA incubation for 24 h. The BCA protein assay kit (Pierce) was used to normalize the cytoplasmic protein. The protein was stored in -80˚C until it was analyzed by cytochrome C ELISA kit according to the manufacturer's instructions.

Western blotting and immunofluorescent analysis. Western blotting was performed as described previously (22). The following antibodies were used: antibodies against PI3K, Akt, phospho-Akt, GSK-3β, phospho-GSK-3β, NF-κB phospho-p65, JNK, phospho-SAPK/JNK, phospho-MDM2, IκB (1:1000, Cell Signaling Technology); NF-κB p65, phospho-IκB (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and actin (1:1000, Thermo Scientific IHC, Fremont, CA).

The immunofluorescent analysis was performed as previous protocol (23). After UA treatments, the MIA PaCa-2 cells were fixed in 4% paraformaldehyde for 30 min and perforated by PBS containing 0.1% Triton X-100. The cells were blocked with 5% bovine serum albumin (BSA) for 1 h at 37˚C and incubated the NF-κB p65 antibody (1:200) at 4˚C overnight. After washing by PBS, Cy3-conjugated goat anti-rabbit IgG (1:50, Proteintech, Chicago, IL) was used as the secondary antibody for 1 h at 37˚C. Nuclear staining was achieved by incubating the cells in DAPI for 10 min. The fluorescence was photographed with fluorescence microscopy (Olympus).

Mouse xenograft model and immunohistochemical analysis (IHC). To establish a subcutaneous xenograft model, MIA PaCa-2 cells ($5x10^6$) were inoculated subcutaneously on the flanks of 6-week-old nude mice (female, purchased from Experimental Animal Center of Southern Medical University). When the average tumor size reached ~100 mm^3, mice with tumor xenografts were randomized into three groups with six mice in each group including: i) vehicle alone (normal saline with 1% DMSO i.p.), ii) low dose UA (100 mg/kg twice a week i.p.), iii) high dose UA (200 mg/kg twice a week i.p.). All of the treatments were administered at a dose of 0.1 ml/10 g bodyweight. The formula, (length x width²)/2, was used to estimate tumor sizes. Both the body weight and tumor sizes were recorded twice a week. At the end of the experiment, the animals were sacrificed and the tumors were dissected and weighed to calculate the inhibitory rate. Animal studies were performed according to the institutional guidelines of Experimental Animal Center in Sun Yat-sen University.

The paraffin tissue sections were obtained from the tumor tissues from the nude mice, IHC were performed as described previously (24). Briefly, the sections were deparaffinized with xylene and microwaved for 10 min in the presence of 10 mM citric acid buffer. After quenching the endogenous peroxidases by hydrogen peroxide, the sections were blocked by 5% BSA/0.3% Triton X-100 for 20 min. Then the sections were incubated with cleaved caspase-3 (1:200) antibody overnight at 4˚C and secondary antibody, goat anti-rabbit IgG (1:50, Proteintech) for 30 min at 37˚C, and then signal was generated by using the DAB substrate kit (Boster, Wuhan, China) for peroxidase.

Statistical analysis. Data are expressed as mean ± standard deviation (SD) of three separate experiments and analyzed by SPSS. $P<0.05$ was considered significant and determined by the two-sample Student's t-test or one-factor ANOVA analysis.
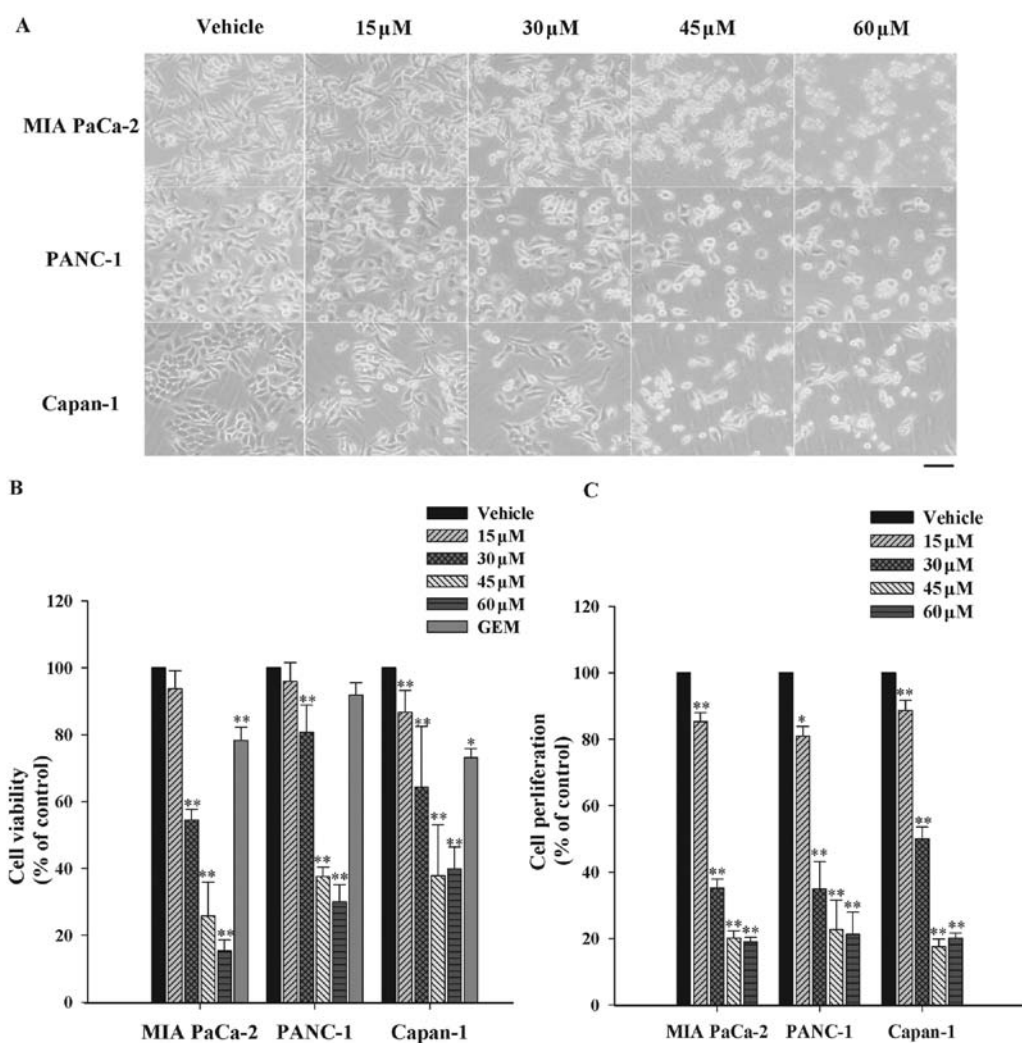
Figure 1. Ursolic acid (UA) dose-dependently decreases the cell viability and proliferation in three different malignancy grades of pancreatic resistant cancer cell lines (MIA PaCa-2, PANC-1 and Capan-1). (A) The effect of UA on cell morphology. The cells were treated with 15, 30, 45 and 60 μM UA for 24 h. Scale bar, 50 μm. (B) The effect of UA on cell viability by MTT assay. (C) The effect of UA on cell proliferation by the colorimetric 5'-bromo-2'-deoxy-uridine (BrdU) incorporation assay. Mean ± SD (n=3). **P<0.05 vs vehicle; **P<0.01 vs vehicle.

Results

Ursolic acid (UA) decreases the viability and proliferation of the human pancreatic resistant cancer cell lines. In order to appraise the cytotoxicity of UA on pancreatic-resistant carcinoma, three different malignancy grades of pancreatic resistant cancer cell lines including MIA PaCa-2 (grade 3), PANC-1 (grade 2) and Capan-1 (grade 1) were exposed to increasing concentrations of UA (15, 30, 45 and 60 μM) for 24 h (6,25-27). Firstly, the morphology of three cell lines under microscopic observation revealed the corresponding reduction of surviving cell numbers and morphological changes after UA treatments (Fig. 1A). In addition, cell viability was analyzed by MTT assay. As shown in Fig. 1B, UA dose-dependently decreased the viability of three cell lines with the half maximal inhibitory concentration (IC_{50} value) at 40.8, 45.3 and 41.3 μM, respectively. Gemcitabine as the positive control at the concentration of 45 μM did not induce significant cytotoxicity to PANC-1 cells comparing with the vehicle for 24 h. However, UA decreased the viability by 62.5% at the same concentration in the identical cell lines. Similarly,

UA achieved stronger cytotoxic effect than gemcitabine in MIA PaCa-2 and Capan-1 cells. The proliferation inhibition of UA was further investigated by 5'-bromo-2'-deoxy-uridine (BrdU) incorporation assay. As observed in Fig. 1C, growth inhibition effect of UA in MIA PaCa-2 cells started at 15 μM and increased up to 60 μM. The results were in agreement with the outcomes of MTT assay. These data suggested that UA was capable of causing growth inhibition to pancreatic-resistant carcinoma *in vitro*.

Both intrinsic and extrinsic pathways are involved in UA-triggered apoptosis. UA has been shown to induce apoptosis in multiple cancer cell lines (16-20). To identify whether UA has the same effect in pancreatic-resistant cancer cell lines, the morphologic analysis was performed by Hoechst 33258 staining. MIA PaCa-2, PANC-1 and Capan-1 cells were exposed to various concentrations (15, 30, 45 and 60 μM) of UA for 24 h, the morphological characteristics of apoptotic cells such as condensed nuclei and cell shrinkage were discovered and apoptotic proportion grew with upward dose (Fig. 2A). To confirm the UA-triggered apoptosis in three cell lines, another
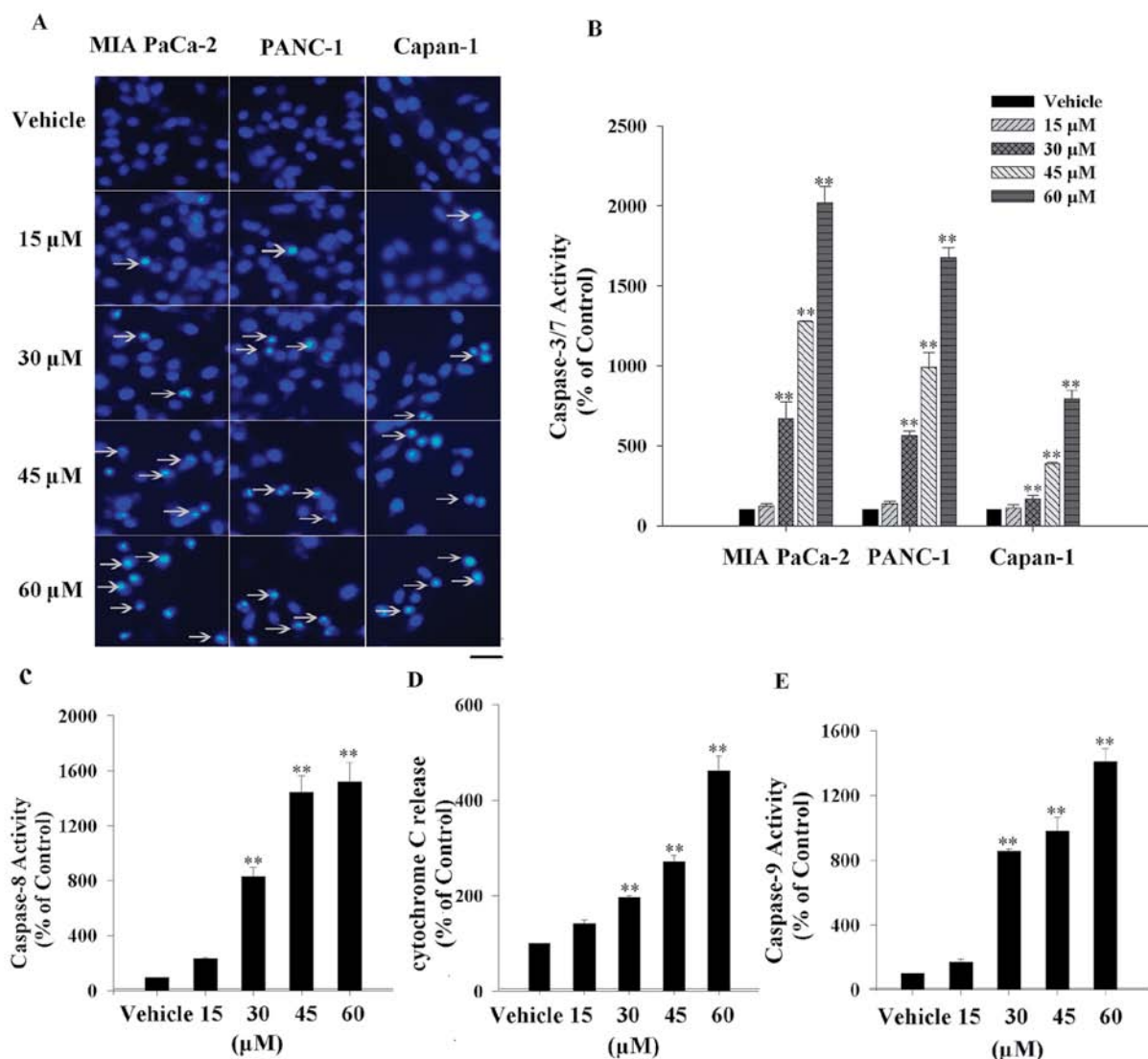
Figure 2. Ursolic acid (UA)-triggered apoptosis is mediated via the intrinsic and extrinsic pathways in pancreatic resistant cancer cell lines. (A) Hoechst 33258 staining. MIA PaCa-2, PANC-1 and Capan-1 cells were incubated with 15, 30, 45 and 60 μM UA for 24 h. Scale bar, 50 μm. Arrows indicate the nuclei of apoptotic cells. (B-E) The effect of UA on (B) the caspase-3/7, (C) the caspase-8 activity, (D) the cytochrome C release and (E) the caspase-9 activity in MIA PaCa-2 cells. Mean ± SD (n=3). **P<0.01 vs vehicle.

apoptotic marker, caspase-3/7 activity, was examined. Clearly, the caspase-3/7 activity was promoted gradually along with the rising concentrations of UA. Fig. 2B shows that the caspase-3/7 activity was enhanced 20-, 16- and 8-fold, respectively, in MIA PaCa-2, PANC-1 and Capan-1 cells at 60 μM UA.

The caspase-dependent cascade is mediated by endogenous and exogenous signal transductions (22). To study by which UA induces caspase-3/7 activation, several markers belonging to the two pathways were inspected. Caspase-8 is a downstream apoptotic molecule of the death receptor, which mediates the death receptor signaling pathway (extrinsic pathway). As shown in Fig. 2C, caspase-8 activity increased by ~15-fold with 60 μM UA treatment. Cytochrome C released from mitochondria into cytoplasm was the initial step in the intrinsic apoptotic pathway. Fig. 2D exhibits the cytochrome C relative release rate increase by ~4.5-fold at 60 μM UA. Besides, caspase-9 activity, another marker of the intrinsic apoptotic pathway, was also increased by ~14-fold after 60 μM UA intervention (Fig. 2E).

c-Jun-terminal kinase (JNK) pathway is potentially involved in UA-induced endogenous apoptosis. The mitogen-activated protein kinase (MAPK) family is closely related to cell apoptosis (28). There are some reports revealing that UA is able to activate JNK, which is one of crucial components in MAPK signaling pathway (16,29). Here, we detected JNK and P38 expression levels, both of which were reported as vital molecules of MAPK family in the programmed cell death, to explore more potential signal channels (30). We found that JNK activity, measured by phosphorylated-JNK level, was significantly up-regulated at the presence of 30 and 45 μM UA. But, UA had no obvious effect on JNK, P38 and phosphorylated-P38 protein levels (Fig. 3A). This result indicated that JNK may contribute to UA-induced apoptosis. To further study the apoptotic pathway in which JNK was involved, we used the JNK inhibitor SP600125 to block the activity of JNK pathway. MIA PaCa-2 cells were pretreated with 10 μM SP600125 for 1 h, and then treated with 45 μM UA for 12 h. As shown in Fig. 3B and C, pretreatment
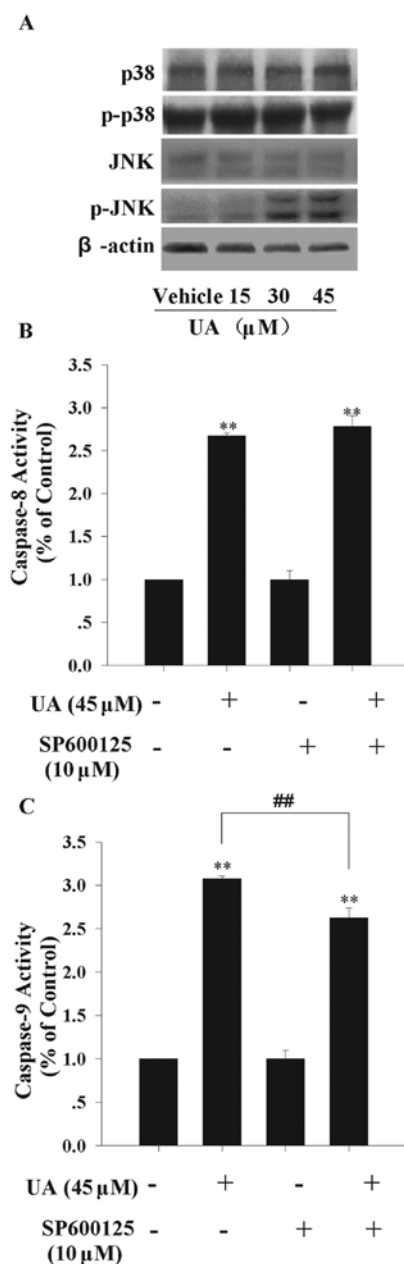
Figure 3. c-Jun-terminal kinase (JNK) pathway is potentially involved in ursolic acid (UA)-induced endogenous apoptosis in MIA PaCa-2 cells. (A) The effect of UA on the protein levels of JNK, phosphorylated-JNK, P38 and phosphorylated-P38 by western blotting. MIA PaCa-2 cells were incubated with 15, 30, 45 μM UA for 12 h. (B) The effect of 45 μM UA on the caspase-8 and (C) the caspase-9 activity at 12 h after the pretreatment of the JNK inhibitor SP600125 (10 μM) for 1 h. Mean ± SD (n=3). **$P<0.01$ vs vehicle; ##$P<0.01$ vs UA 45 μM.

by SP600125 partially inhibited the caspase-9 activity by 16%, while caspase-8 activity was not altered significantly. These data indicated that JNK is at least partly involved in UA-induced endogenous apoptotic signaling transduction.

UA inhibits the PI3K/Akt pathway and down-regulates the expression of downstream proteins. To ascertain the potential mechanism by which UA caused growth suppression and apoptosis to pancreatic cancer, we also examined the activity of PI3K/Akt pathway in MIA PaCa-2 cell line. When cells were in the presence of increasing concentrations of UA (15,

30 and 45 μM) for 12 h, the results by western blotting demonstrated the total PI3K and the phosphorylated Akt levels were attenuated gradually in a dose-dependent manner, while the total Akt protein expressions did not alter (Fig. 4A). PI3K/Akt pathway is constitutively activated in most tumor cell lines and regulates a series of biological characteristics of tumors such as growth and apoptosis (31). Therefore, we checked the possible Akt targeting effector molecules downstream. Similar changes to Akt, were recorded for the phosphorylation level of GSK-3β, MDM2, P65, IκB that also decreased gradually, whereas the total proteins of GSK-3β, P65, IκB did not show any corresponding alteration (Fig. 4B).

PI3K/Akt/NF-κB pathway is involved in UA-induced cell viability decrease, but not intrinsic and extrinsic apoptosis. Aberrant activation of NF-κB greatly contributes to chemoresistance in pancreatic cancer, and suppression of NF-κB sensitizes the treatment outcome of gemcitabine (32). To assess whether the UA-induced NF-κB activation is a downstream event of PI3K/Akt signaling pathway, IκB and its phosphorylated form were further investigated. We inhibited the phosphorylation of Akt by 10, 20, 40 μM LY294002 for 12 h. As seen in Fig. 4C, 20 μM LY294002 and above abolished more than half of the phosphorylated level of Akt protein. Phosphorylated IκB was evidently modulated downward in a dose-dependent pattern. However, there was no alteration of total IκB within 12 h. In addition, the nuclear translocation of the cytoplasmic NF-κB p65 subunit was also checked by immunofluorescence. UA (45 μM) and/or 40 μM LY294002 for 12 h repressed the NF-κB p65 nuclear translocation compared with vehicle (Fig. 4D). These results suggested there was crosstalk between PI3K/Akt and NF-κB signaling pathways in MIA PaCa-2 cells.

We next examined the involvement of PI3K/Akt/NF-κB pathway in the apoptotic cascade. The MIA PaCa-2 cells were incubated with 40 μM LY294002 for 12 h. As seen in Fig. 4E and F, inhibiting the activation of Akt can not activate caspase-8 and -9. Hence, PI3K/Akt/NF-κB pathway is not the main participant in UA-induced intrinsic and extrinsic apoptotic signaling transductions. However, the cell viability was decreased by ~25% after exposure to 40 μM LY294002 for 24 h. These findings indicated that PI3K/Akt/NF-κB pathway was involved in UA-induced cytotoxicity to MIA PaCa-2 cells, but the mechanism was not caspase-8- and -9-dependent.

UA overcomes chemoresistance in other resistant cancer cell lines. We also tested whether UA possessed the potential to induce other resistant cancer cells death in the similar fashion. A paclitaxel-resistant endometrial cell line (HEC-1A) and a platinum-resistant ovarian cancer cell line (OVCAR-3) were used to examine the cell viability after UA exposure (33,34). As seen in Fig. 5A and B, UA inhibited the cell viability in a time and dose-dependent manner. Moreover, UA also increased the phosphorylated JNK, and decreased the expression of phosphorylated Akt (Fig. 5C and D). These results further confirmed that the mechanism of action of UA was associated with JNK and Akt pathways.

UA causes tumor regression in MIA PaCa-2 xenograft model. The data described above clearly showed that UA was a potent compound to inhibit proliferation and caused deaths of various
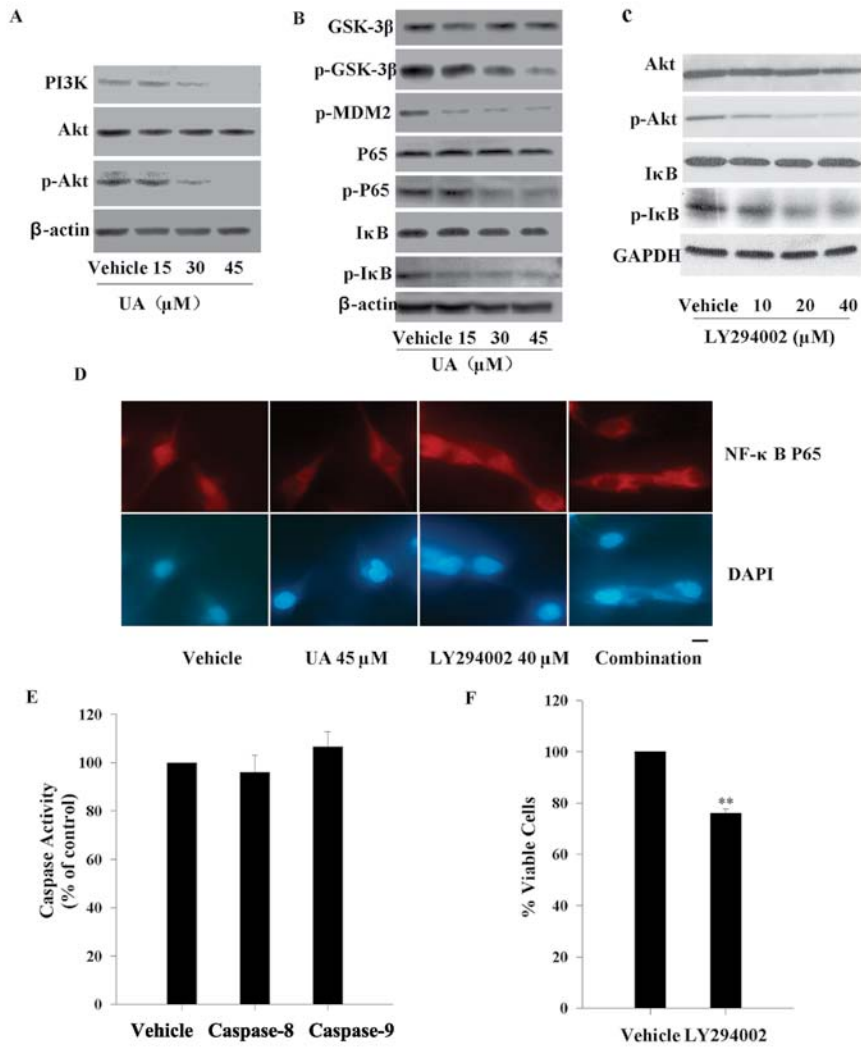
Figure 4. PI3K/Akt/NF-κB pathway is involved in ursolic acid (UA)-induced cell viability decreases, but not intrinsic and extrinsic apoptosis in MIA PaCa-2 cells. (A) The effect of UA on the protein levels of PI3K, Akt and phosphorylated-Akt by western blotting. MIA PaCa-2 cells were incubated with 15, 30, 45 μM UA for 12 h. (B) The effect of UA on the downstream protein levels of PI3K/Akt pathway at 12 h. (C) The effect of Akt inhibitor LY294002 on IκB and phosphorylated-IκB expressions by western blotting. MIA PaCa-2 cells were treated with 10, 20, 40 μM LY294002 for 12 h. (D) The effect of Akt inhibitor 40 μM LY294002 on NF-κB p65 nuclear translocation at 12 h. (E) The effect of 40 μM LY294002 on the caspase-8 and -9 activity at 12 h. Scale bar, 10 μm. (F) The effect of 40 μM LY294002 on the cell viability at 24 h. Mean ± SD (n=3). **P<0.01 vs vehicle.
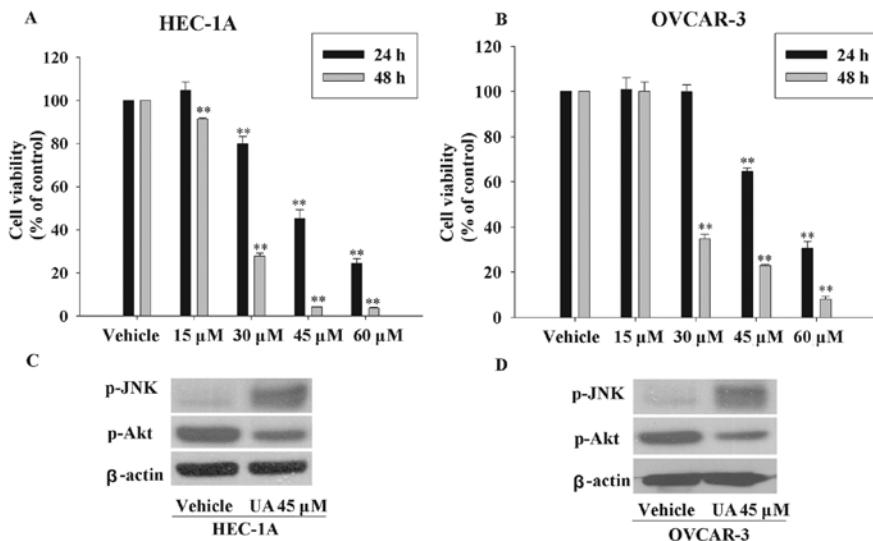

Figure 5. Ursolic acid (UA) could overcome chemoresistance in other resistant cancer cell lines. (A and B) The effect of UA on cell viability in HEC-1A and OVCAR-3 cell lines. The two cell types were treated with 15, 30, 45, 60 μM UA for 24 h and the viability was examined by MTT assay. (C and D) The effect of UA on the protein levels of phosphorylated-JNK, and phosphorylated-Akt by western blotting. Mean ± SD (n=3). **P<0.01 vs vehicle.
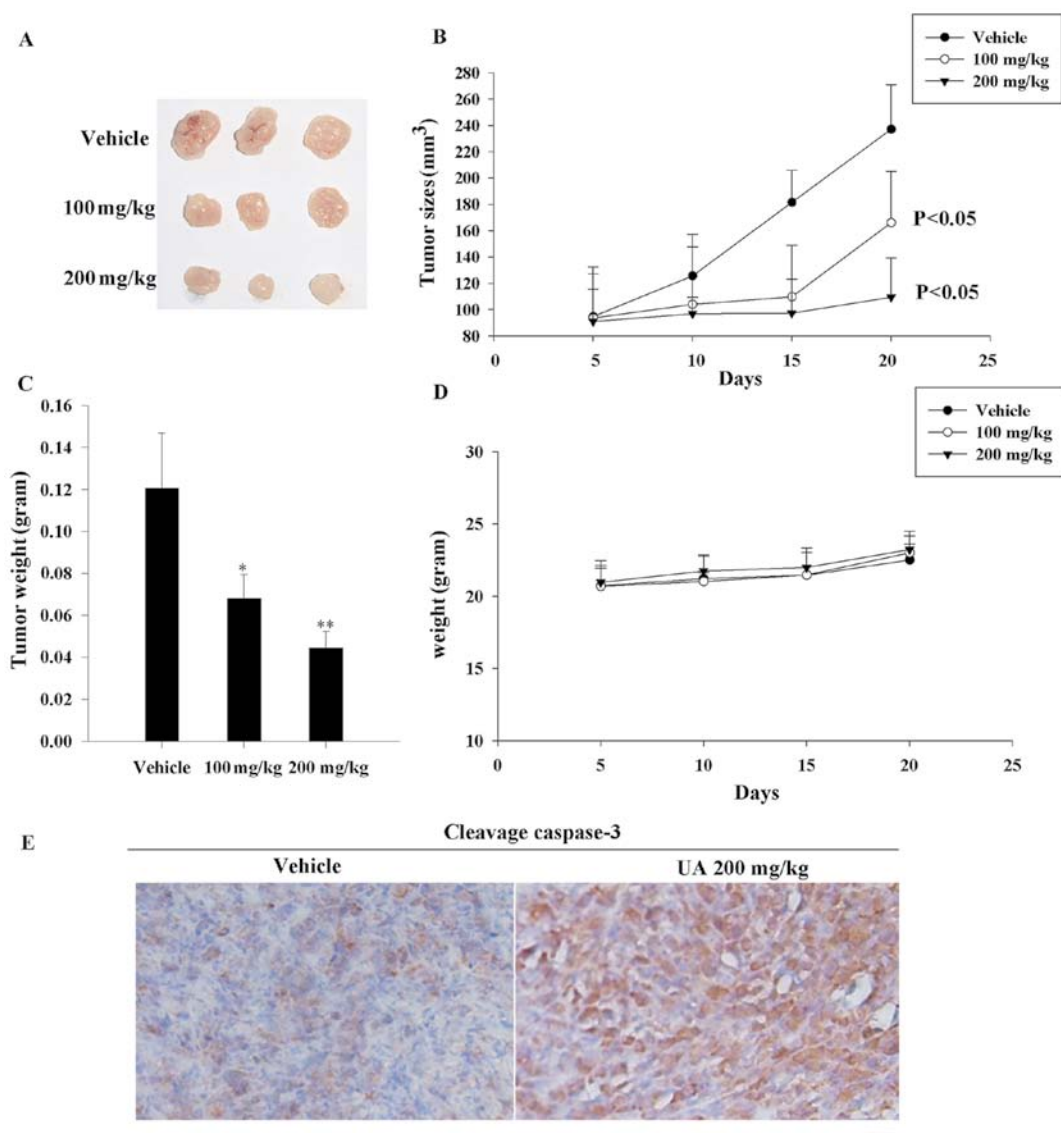
Figure 6. Ursolic acid (UA) suppresses the gemcitabine-resistant pancreatic cancer *in vivo*. (A) UA caused pancreatic tumor regression *in vivo*. MIA PaCa-2 cells ($5x10^6$) were injected into the flanks of 6-week-old nude mice. When the average tumor size reached ~100 mm³, mice were randomly divided into three groups with six mice in each group and treated with saline with 1% DMSO, UA at 100 and 200 mg/kg twice a week, respectively. (B) The growth curve of xenografts in mice. (C) The tumor weight of xenografts in mice after the end of UA treatment. (D) The body weight curve of mice to evaluate the toxicity of UA. (E) The effect of UA on activity of the cleavage caspase-3 *in vivo* by immunohistochemistry. Scale bar, 50 μm. Mean ± SD. *P<0.05 vs vehicle; **P<0.01 vs vehicle.

chemotherapy-resistant cancer cell lines. Thus we tried to apply UA in a nude mouse xenograft model to provide evidence for clinical application. Until the tumor volume reached ~100 mm³, the mice were treated i.p. with either vehicle or UA at 100/200 mg/kg twice a week. Significant inhibition of tumor growth by UA was observed after UA treatment for 20 days (Fig. 6A and B). Tumor weights in UA-treated groups were also lower than the vehicle group (30% less in 100 mg/kg group and 50% less in 200 mg/kg group) (Fig. 6C). In order to measure the safety of UA, body weights of mice were regularly detected. As seen in Fig. 6D, there were no significant differences compared with control group after the UA treatment. Furthermore, we checked the cleavage caspase-3 expression by immunohisto-chemistry to reveal that the tumor in UA-exposed group had more visible apoptosis. These data elucidated that UA caused resistant pancreatic cancer death also *in vivo*.

Discussion

Due to the poor early diagnosis and serious chemotherapy resistance, pancreatic carcinoma is highly fatal. Although gemcitabine is known as the most active chemotherapy agent for this tumor, it has only a slight survival benefit and shows an objective tumor response rate of <10% owing to pre-existing or acquired chemo-resistance (35,36), thus demanding urgent development of effective therapeutic options. Here we demonstrate that a small molecule ursolic acid (UA), a well known anti-oxidative and anti-inflammatory agent, significantly suppresses the growth and induces apoptosis of resistant pancreatic cancer through activation of c-Jun-terminal kinase (JNK) pathway and inhibition of PI3K/Akt/NF-κB pathway.

UA, belonging to pentacyclic triterpenoid, is a soluble, natural, and small molecule compound, which has displayed

its proliferation-inhibiting and apoptosis-inducing effects in some chemotherapy-resistant tumors (16,17,37). Previous study showed that UA only decreased the cell viability by ~40% in a hormone refractory prostate cancer cell line at 72 h (16). In contrast to the prostate cancer cells, UA exhibits much more potent cytotoxicity to pancreatic tumors. The half maximal inhibitory concentration (IC_{50} value) of UA was 40.8 μM in highly malignant pancreatic cancer cells (MIA PaCa-2 cells) within 24 h *in vitro*. In our data, both modulation of proliferation and induction of apoptosis by UA were observed within the time course of 24 h. However, UA had suppressed the proliferation significantly at 15 μM rather than any type of caspase activity or cytochrome C relative release rate. The alterations of proliferation inhibiting rate and caspase-3/7 activity were detected at 30-45 μM UA. Furthermore, UA at the higher concentration (60 μM) was only perceived to be effective in apoptosis induction. Hence, the results seem to indicate that decreased proliferation and increased apoptosis by UA together impact cell viability of pancreatic cancer cells.

The JNK pathway reported as important apoptosis inducing factor exerts antagonistic effects on apoptosis (30). JNK, a mitogen-activated protein kinase (MAPK), can be activated primarily by exposure to environmental stress (38). Although the extracellular signal-regulated kinase (ERK) pathway is considered as a great contributor to oncogenesis, previous study has demonstrated that it plays a lesser role in mitogen-induced survival of pancreatic cancer (39). However, many studies have reported that JNK pathway plays a crucial role in UA-induced apoptosis in many cancers (16,29,40). In our study, the activation of JNK, but not P38, was induced by UA in a dose-dependent manner in MIA PaCa-2 cells. Two major apoptotic signaling transductions, namely the receptor-mediated/exogenous and mitochondrial/endogenous pathways, are activated in UA-induced apoptosis in MIA PaCa-2 cells. The trigger of endogenous pathway depends on the release of cytochrome C from mitochondria to cytosol and activation of caspase-9 (41). In the extrinsic pathway, the binding of death ligands to their homologous receptors drives activity of caspase-8 (42). Here, we showed that SP600125, a specific JNK inhibitor, could partly protect UA-induced caspase-9 activation rather than caspase-8. Our data are consistent with a previous report (16). Teraishi *et al* (43) have proved that JNK activation is required for gemcitabine to achieve its cytotoxicity and the weaker activity of JNK may result in gemcitabine resistance. Our data displayed that JNK pathway was activated excessively in the gemcitabine resistant cell line (MIA PaCa-2). We proved similarly that JNK pathway participated in UA-induced mitochondrial apoptotic signaling transduction in MIA PaCa-2 cells. Thus we speculate that UA-induced JNK activation may contribute to overcome gemcitabine resistance and induces programmed cell death in gemcitabine resistant pancreatic cancer.

The current consensus to PI3K/Akt signaling transduction is that this cascade contributes greatly to tumor progression and chemotherapy resistance (44). PI3K is a broadly expressed lipid kinase, the main action of which is to execute the catalyzed reaction to synthesize the second messenger-PIP_3 from PIP_2 (44,45). Akt, acting downstream of PI3K, is usually activated by directly binding to PIP3 through its pleckstrin-homology (PH) domain and phosphorylation (44). Akt activation is closely relevant to anti-apoptosis and proliferative signal transduction. Primarily, Akt stimulates the degradation of IκB which results in the nuclear translocation of P65 and activation of target genes (46). Akt also catalyzes phosphorylation of MDM to improve its function to bind to P53 and enhance p53 degradation (47,48). In the aspect of cell proliferative promotion, Akt inhibits the kinase activity of GSK3β by maintaining the phosphorylated level to protect cyclin D from degradation (49). Our results showed that UA strongly suppressed the activation of Akt and blocked the activity of its downstream molecules mentioned above.

We proved that suppressing the Akt activity by LY294002, a selective Akt inhibitor, down-regulated the NF-κB activation by reducing the p65 nuclear translocation and phosphorylating IκB in MIA PaCa-2 cells. We propose that PI3K/Akt pathway seems crosstalk with NF-κB pathway in pancreatic cancer cells. Arlt *et al* (32) has reported previously the evident correlation between basal NF-κB activity and gemcitabine resistance in pancreatic cancer cells. There is some evidence showing that PI3K/Akt inhibition promoted anti-tumor effectiveness of gemcitabine via inhibiting PI3K/Akt phosphorylation *in vitro* and *in vivo* (50,51). UA may demonstrate its potent anti-cancer effects via PI3K/Akt/NF-κB pathway in MIA PaCa-2 cells. We observed that LY294002 did not protect MIA PaCa-2 cells from caspase-8/9 activations. Therefore, exogenous and endogenous apoptosis are not affected equally by UA-triggered Akt activation. However, LY294002 decreased the viability of MIA PaCa-2 cells to some extent, and it is well-known that cell viability is a combination of cell proliferation and death. We argue that the UA-induced PI3K/Akt/NF-κB pathway suppression may only affect the pancreatic cancer cell proliferative rate. Besides, recent study also demonstrated that UA was able to induce tumor cells apoptosis through a caspase-independent pathway (17). That phenomenon means UA has the chance to apply for combined treatment to overcome chemotherapy resistance. In our study, we can not exclude that the PI3K/Akt/NF-κB pathway also triggers caspase-independent apoptosis. But further studies are needed to prove the hypothesis.

To examine whether the effect of UA on JNK and Akt pathway is confined to resistant pancreatic cancer cells solely or is a general phenomenon which is applicable to other resistant cancer cells, we examined the effect of UA on an endometrial cell line (HEC-1A) and an ovarian cancer cell line (OVCAR-3) because both of them are highly resistant to chemotherapy. According to our data, the JNK activity was inhibited, but Akt was excessively activated in these cells. UA significantly decreased the cell viability of HEC-1A cells and OVCAR-3 cells, which was associated with an increase of JNK activation and a decrease of Akt activation. For future clinical application, the safety and efficacy of UA were further detected *in vivo*. We presented evidence that UA suppressed tumor growth without any obvious side effects. As a natural compound extracted from edible foods and herbs, UA is relatively low-toxic with LD_{50} value >637 mg/kg intraperitoneally in mice. Our studies provide further evidence that UA is a potent compound to apply to overcome chemoresistance in oncological therapy.

In conclusion, UA is a small, safe, potent and multifuntional molecule which is easily produced and relatively safe. UA has been applied in clinical trial in some areas to treat cancer (52). Consequently, UA is comparable or even superior to conventional anti-tumor drugs, encouraging further the use of this small

molecular compound in gemcitabine resistant pancreatic cancer and other chemo-insensitive cancers.

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