

Oridonin overcomes the gemcitabine resistant PANC-1/Gem cells by regulating GST pi and LRP1/ ERK/JNK signalling

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Background: Chemotherapy remains a primary treatment method for advanced pancreatic cancer. However, chemotherapy resistance can influence the therapeutic effect of pancreatic cancer. The resistance mechanism of chemotherapeutic agents such as gemcitabine, which is an agent typically used to treat pancreatic cancer, is complicated and can be influenced by genes and the environment. Oridonin is a tetracyclic diterpenoid compound extracted from the traditional Chinese herb *Rabdosia labtea*. Oridonin may overcome drug resistance in pancreatic cancer, but researching pancreatic cancer drug resistance of chemotherapy by oridonin is not completely understood.

Purpose: The present study aimed to assess the impact of oridonin on multidrug resistance proteins, apoptosis-associated proteins and energy metabolism in gemcitabine-resistant PANC-1 (PANC-1/Gem) pancreatic cancer cells.

Methods: Gemcitabine resistance in PANC-1/Gem cells was induced using a concentration gradient of gemcitabine. Cell Counting Kit-8 assays were used to detect the impact of gemcitabine and oridonin on the proliferation of PANC-1 and PANC-1/Gem cells. Western blot analysis and immunofluorescence were used to detect the expression of multidrug resistance proteins, apoptosis-associated proteins and low-density lipoprotein receptor protein 1 (LRP1) proteins in PANC-1/Gem cells. The effects of gemcitabine and oridonin on PANC-1/Gem cells apoptosis were detected using flow cytometry. Animal xenograft tumor assays were used to detect the effect of gemcitabine and oridonin on pancreatic cancer in vivo. Furthermore, the ATP Assay kit was used to determine the effects of gemcitabine and oridonin on ATP levels in PANC-1/Gem cells. Immunofluorescence assays were used to detect the effects of gemcitabine and oridonin on the expression of low-density lipoprotein receptor protein 1 (LRP1) in PANC-1/Gem cells. In addition, LRP1 expression was knocked down in PANC-1/Gem cells via lentiviral vector-mediated RNA silencing. Clone formation assays and Western blot analysis were used to detect the effect of LRP1 knockdown on the proliferation of PANC-1/Gem cells.

Results: The present results demonstrate that oridonin overcomes PANC-1/Gem cells gemcitabine resistance by regulating GST pi and LRP1/ERK/JNK signaling.

Conclusion: In conclusion, the present study indicated that oridonin could overcome gemcitabine resistance in PANC-1/Gem cells by regulating GST pi and LRP1/ ERK/JNK signaling, inducing cell apoptosis. Therefore, oridonin with gemcitabine may be a promising preoperative treatment for patients who suffer from pancreatic cancer.

Keywords: oridonin, gemcitabine resistance, pancreatic cancer PANC-1 cells, glutathione S-transferase pi, low density lipoprotein receptor

Introduction

In previous years, the incidence of pancreatic cancer has increased worldwide. Notably, the 5-year survival rate is low (5%) and the median survival is <6 months due to its poor prognosis.¹ Furthermore, 80% of patients who are diagnosed with pancreatic cancer have distant metastases. Typically, patients with advanced pancreatic cancer respond unfavorably to surgical treatment. Therefore, chemotherapy remains the primary treatment method for advanced pancreatic cancer. The chemotherapeutic agent gemcitabine has been approved by the US Food and Drug Administration as a first-line therapy for pancreatic cancer since 1997.² Multiple studies have demonstrated the improved effects of gemcitabine treatment compared with 5-fluorouracil in pancreatic cancer. However, gemcitabine treatment also has some disadvantages, including its association with multiple adverse events and chemoresistance.^{3–5} It has been suggested that improving the responsiveness to gemcitabine in pancreatic cancer may increase patient survival. Therefore, identifying an agent to overcome gemcitabine resistance in pancreatic cancer may be a potential method to improve the treatment of pancreatic cancer in clinical settings.

Through the holistic view and syndrome differentiation and treatment, traditional Chinese medicine (TCM) takes the approach of multitarget and overall-regulation to treat tumors.⁶ Traditional Chinese medicine (TCM) is regarded as an important treatment for malignancies, especially for those in advanced stage.⁷ Oridonin, a traditional Chinese medicine extracted from *Rabdosia rubescens*, has been indicated to promote inhibitory effects on a variety of tumors.^{8,9} Our previous studies revealed that oridonin could inhibit the growth of human pancreatic cancer cells by increasing apoptosis, downregulating the expression of the mRNA inflammation and inhibiting cell migration.^{10,11} However, to the best of our knowledge, it has not yet been reported whether oridonin can overcome drug resistance in pancreatic cancer.

The aim of the present study was to demonstrate whether oridonin could overcome the drug resistance in pancreatic cancer. Our study showed that resistance proteins and low-density lipoprotein receptor protein 1 (LRP1) proteins were down expression after treatment with oridonin. Furthermore, we found that oridonin could induce cell apoptosis and inhibited tumor growth. Therefore, the findings of the present study may have potential clinical applications for pancreatic cancer treatment.

Materials and methods

Cell lines and cell culture

Human pancreatic cancer PANC-1 cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). PANC-1 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO₂ at 37° C.

Induction of PANC-1/Gem cells

PANC-1 cells (1×10^5) were cultured in 25 cm² flasks for 24 h and then exposed to 0.5 μM gemcitabine for 72 h. Surviving cells were cultured in drug-free DMEM medium to allow cells to reach 80% confluence. The cells were cultured at this drug concentration until they grew steadily. The gemcitabine concentration was continuously increased in the culture medium for a total of 6 months until PANC-1/Gem cells were established. PANC-1/Gem cells were grown in drug-free medium for 2 weeks and subsequently harvested, frozen in the liquid nitrogen and stored at -8° C until analysis. PANC-1/Gem cells were cultured in drug-free medium for 2 weeks prior to performing the experiments.

Cell cytotoxicity and proliferation assay

Cell viability was quantified using the Cell Counting Kit-8 (CCK-8) kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. PANC-1 (1×10^5 cells/ml, 100 μl/well) and PANC-1/Gem (5×10^4 cells/ml, 100 μl/well) cells were seeded into 96-well plates and cultured for 24 h, respectively. Following this, the medium was replaced with 100 μl culture medium containing different concentrations of oridonin (0, 10, 20, 40, 80 or 160 μM), which was obtained from Gracia Chemical Technology Co., Ltd., or gemcitabine (0, 1, 2, 4, 8 or 16 μM). Cells were incubated for a total of 24 h. Negative control cells were treated with medium containing 0.1% dimethyl sulfoxide (DMSO) only. The same concentration of DMSO was used for drug preparation. Following oridonin treatment for 24 h, 10 μl of CCK-8 solution was added into each well and the cells were incubated for 4 h at 37°C. The absorbance was measured at 450 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the percentage of cell viability was calculated as follows: Viability ratio% = $A_{450(\text{oridonin})} / A_{450(\text{control})} \times 100\%$. The

IC₅₀ value was calculated as the concentration of oridonin that inhibited cell growth by 50%. At least three independent experiments were performed.

Western blot analysis

PANC-1 and PANC-1/Gem treated with oridonin or gemcitabine were collected after 24-h incubation, rinsed twice with phosphate-buffered saline (PBS) and lysed in radioimmuno-precipitation assay lysis buffer (Beyotime Institute of Biotechnology) with phenylmethylsulfonyl fluoride. The lysates were centrifuged, and the supernatants were collected to be quantitated using a bicinchoninic acid assay kit [cat. no. PQ0011; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China]. A total of 50 µg of protein was loaded onto 12% SDS-polyacrylamide gels for electrophoresis and transferred to polyvinylidene difluoride membranes. Blocking was performed with 5% skimmed milk in Tris-buffered saline with Tween-20 for 2 h at room temperature. Following this, the membrane was incubated overnight at 4° C with the following primary antibodies: Anti-P glycoprotein (P-gp; cat. no. ab170904), anti-glutathione S-transferase (GST)3/GST pi (cat. no. ab138491), anti-multidrug resistance protein 1 (MRP1; cat. no. ab180960), anti-major vault protein (MVP; cat. no. ab175236), anti-topoisomerase II alpha (Topo2; cat. no. ab52934), anti-Caspase 3 (cat. no. ab13847), anti-Caspase 9 (cat. no. ab32939), anti-B-cell lymphoma-2 (Bcl-2; cat. no. ab32124), anti-Bcl-2-associated protein (Bax; cat. no. ab32503), anti-low-density lipoprotein receptor 1 (LRP1 cat. no. ab92544), anti-c-Jun N-terminal kinase (JNK)1/2/3 (cat. no. ab124956), anti-Phospho-JNK1/2/3 (cat. no. ab219584), anti-extracellular signal-regulated kinase (ERK)1/2 (cat. no. ab184699), anti-phospho-ERK1/2 (cat. no. ab184699), anti-nuclear factor (NF)-κB p105/p50 (cat. no. ab32360; all Abcam, Cambridge, MA, USA); and anti-β-actin [cat. no. Mab1445; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.]. The membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase: Goat anti-mouse IgG [cat. no. GAM0072; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.] or goat anti-rabbit IgG [cat. no. GAR0072; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.] for 2 h at room temperature. The membranes were then visualized using an ECL substrate kit [cat. no. P1425; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.] on the Omega Lum G imaging system. β-actin was used as the loading control. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify band intensities. All analyses were conducted in triplicate.

Flow cytometry analysis of cell apoptosis

For apoptosis assays, PANC-1/Gem cells that received treatment with 15 µM gemcitabine, 60 µM oridonin or combined 4 µM gemcitabine and 40 µM oridonin for 24 h, respectively, were stained using the Annexin V/propidium iodide (PI) apoptosis kit [cat. no. AP101; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.] according to the manufacturer's instructions. Annexin V-fluorescein isothiocyanate (FITC) binding was analyzed using a flow cytometer (Ex=488 nm; Em=530 nm) with a FITC signal detector (usually FL1). PI staining was analyzed using the phycoerythrin emission signal detector (usually FL2). At least 10,000 cells were analyzed for each treatment.

Immunofluorescence

For immunofluorescence experiments, PANC-1/Gem cells were plated onto 35-mm glass-based dishes (cat. no. 801002; Wuxi NEST Biotechnology Co., Ltd., Wuxi, China) 1 day prior to treatment with 15 µM gemcitabine, 60 µM oridonin or combined gemcitabine (4 µM) and oridonin (40 µM) for 24 h. Cells were fixed in 4% poly-formaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 15 min. Following this, cells were blocked with 5% bovine serum albumin for 1 h. Primary antibody incubations with the antibody anti-GST3/GST pi (1:100 dilution) anti-LRP1 (1:100 dilution) and α-tubulin (1:250 dilution; cat. no. ab52866; Abcam) in PBS containing 2% FBS were performed overnight at 4°C. Subsequently, cells were incubated with secondary goat anti-mouse IgG H&L antibody (DyLight 488; 1:400 dilution; ab96871; Abcam) in PBS containing 2% FBS for 2 h at room temperature. Finally, cell nuclei were stained with diamidino-2-phenylindole (0.5 µg/ml) for 15 min, and images were obtained using a fluorescence microscope. PBS was used for all wash steps.

Measurement of intracellular ATP levels

Intracellular ATP levels were measured using an ATP Assay kit (cat. no. KA1661; Abnova Co., Ltd., Taiwan, China). After treatment with gemcitabine, oridonin or combined gemcitabine and oridonin treatment, cells were harvested. Rapid pyrolysis in reaction buffer, take 10 µl of sample add 90 µl reaction mixture, and the sample was analyzed using a GloMax luminometer (GloMax 96 Microplate Luminometer; Promega Corporation; Madison, WI, USA). All analyses were conducted in triplicate.

Lentivirus transduction

LV-LRP1-RNAi and LV-siNC were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). Lentivirus transfections were performed to interfere with LRP1 expression in PANC-1/Gem cells according to the manufacturer's instructions. Briefly, 2×10^5 PANC-1/Gem cells were seeded in each 24-well culture plate for 24 h. Following this, the cells were infected with LV-LRP1-RNAi and LV-siNC in the presence of 5 $\mu\text{g/ml}$ polybrene for a further 24 h. The medium was replaced with 2,000 μl of new culture medium and the cells were cultured for a further 48 h. Knockdown of LRP1 were selected using puromycin in PANC-1/Gem cells.

Clonogenic assay

For the clonogenic assay, 1,000 PANC-1/Gem cells infected with short hairpin LRP1 (shLRP1) or non-silencing control shRNA (shNC). shNC and shLRP1 lentiviruses were seeded in each 6-well culture plate for 15 days. Colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and counted using a stereomicroscope.

Tumor formation assay in nude mice

All animal experiments were performed following Animal Experimental Center of the Zhejiang Chinese Medical University Institutional policies and Apicultural Research of the Chinese Academy of Agricultural Sciences Animal Ethics Committee's guidelines and regulations. A total of 16 BALB/C nude mice (8 male; 8 female; 4 to 5-weeks-old; 20–25 g) were obtained from the Animal Experimental Center of the Zhejiang Chinese Medical University in accordance with Institutional policies. All experimental procedures were approved by the Institute of Apicultural Research of the Chinese Academy of Agricultural Sciences Animal Ethics Committee. The mice were randomly divided into four groups ($n=4/\text{group}$): The control group, the gemcitabine group, the oridonin group, and the combined of gemcitabine and oridonin group. PANC-1/Gem cells (5×10^6), which were suspended in 200 μl DMEM, were subcutaneously injected into the flanks of each mouse. When the tumor volume reached 150 mm^3 , mice received the following treatments: In the gemcitabine group, mice received intraperitoneal injections of 50 mg/kg gemcitabine each week; in the oridonin group, mice received intraperitoneal injections of 10 mg/kg oridonin every day; in the combined gemcitabine and oridonin group, mice received intraperitoneal injections of 25 mg/

kg gemcitabine and 5 mg/kg oridonin each week; and in the control group, mice received intraperitoneal injections of 50 mg/kg saline every day. Tumor sizes and body weights were measured two or three times per week. Tumor volume was calculated as follows: Tumor volume (mm^3) = $\frac{1}{2} \times \text{length} \times \text{width}^2$. After 7 weeks, the mice were sacrificed and xenograft tumors were extracted, weighed, harvested and fixed for the next experiment.

Hematoxylin and eosin (H&E) staining and immunohistochemistry

Tumor sections were fixed in 10% paraformaldehyde and embedded in paraffin. Following this, the samples were cut into 4–6 μm thick sections using a rotation microtome and stained with H&E. Immunohistochemical analysis was performed according to the manufacturer's protocol of Histostain-Plus kits (Bioss; Beijing, China). Sections were incubated with a rabbit anti-GST3/GST pi (1:100 dilution) or anti-LRP1 (1:100 dilution) antibody overnight at 4° C. Following this, sections were incubated with a horseradish peroxidase-conjugated goat-anti-rat secondary antibody (1:100 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at 37°C. Subsequently, the sections were counterstained with hematoxylin and images were captured with an Olympus BX41 microscope.

Calculusyn software

Calculusyn software was used to analyze the drug combination index (CI). $\text{CI}=1$ indicates additive effect; $\text{CI}<1$ indicates synergistic effect. $\text{CI}>1$ indicates antagonist effect.

Statistical analysis

Statistical analysis was performed using SPSS software 19.0 (IBM Corp., Armonk, NY, USA). The results from at least three independent experiments were presented as the mean \pm standard deviation. The statistical significance of the different groups were analyzed using the two-tailed Student's *t*-test or ANOVA. All tests performed were two-sided. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Oridonin inhibits cell proliferation and overcomes gemcitabine resistance in PANC-1/Gem cells

Gemcitabine-resistant cells were established from parental PANC-1 cell lines using exposure to increasing

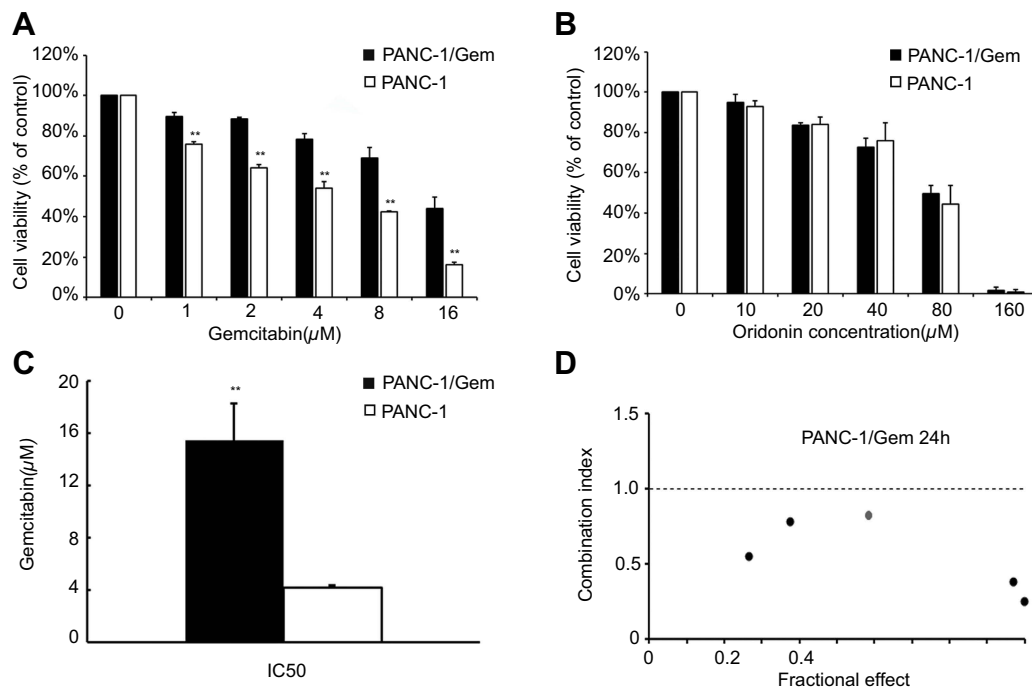


Figure 1 Oridonin inhibits cell proliferation and overcomes gemcitabine resistance in PANC-1/Gem cells. **(A)** PANC-1/Gem and PANC-1 cells were treated with different doses of gemcitabine for 24 h. The cell viability of the indicated cells was determined using CCK-8 assays. $**P < 0.01$. **(B)** Cell viability detected by CCK-8 assays in PANC-1/Gem and PANC-1 cells treated with different doses of oridonin for 24 h. **(C)** IC_{50} values of gemcitabine in PANC-1/Gem and PANC-1 cells. $RI = PANC-1/Gem (IC_{50})/PANC-1 (IC_{50}) = 3.68$. $**P < 0.01$. **(D)** Effect of combination treatment with gemcitabine and oridonin using a constant ratio design. CI of gemcitabine and oridonin treatment in PANC-1/Gem cells was analyzed using Calcsyn software. $CI = 1$ indicates additive effect; $CI < 1$ indicates synergistic effect. $CI > 1$ indicates antagonist effect. **Abbreviations:** CCK-8, Cell Counting Kit-8; RI, reverse index; CI, combination index; IC_{50} , 50% inhibitory concentration.

concentrations (from 0.5 to 8 μM) of gemcitabine over a period of 6 months. The established resistant cells were designated as PANC-1/Gem. The sensitivity of cells to gemcitabine was examined with various concentrations of gemcitabine for 24 h. Compared with PANC-1 cells, PANC-1/Gem cells were indicated to be resistant to gemcitabine (Figure 1A). The IC_{50} of gemcitabine was 15.16 μM in PANC-1/Gem cells and 4.12 μM in PANC-1 cells. In addition, the resistance ratio of PANC-1/Gem cells was 3.68 when compared with PANC-1 cells (Figure 1C). To investigate the effect of oridonin on the viability of PANC-1/Gem and PANC-1 cells, cells were treated with different doses of oridonin for 24 h (Figure 1B). Notably, the IC_{50} of oridonin was 60.23 μM in PANC-1/Gem cells and 57.42 μM in PANC-1 cells. The results suggested that oridonin had no significant impact on the viability of the two cell lines. Calcsyn was used to analyze the drug combination index (CI). The results indicated a synergistic interaction between gemcitabine and oridonin in PANC-1/Gem cell lines, as demonstrated by a consistent CI value of < 1.0 (Figure 1D).

Oridonin regulates the expression of multidrug resistance proteins in PANC-1/Gem and PANC-1 cells

To investigate whether oridonin overcomes gemcitabine resistance in pancreatic ductal adenocarcinoma cells, the effect of gemcitabine and oridonin on multidrug resistance proteins was examined in two cell lines using Western blot analysis. P-gp, MRP1, lung resistance protein/major vault protein (LRP/MVP), Topo2 and GST pi were demonstrated to serve an important role in multidrug resistance(). As indicated in Figure 2A and B, MRP1 and GST pi protein expression levels were significantly suppressed in PANC-1/Gem and PANC-1 cells treated with oridonin compared with the control. However, MRP1 and GST pi protein expression levels were increased after treatment with gemcitabine in the two cell lines. Compared with the gemcitabine treatment group, MRP1 and GST pi activities were decreased when cells were treated with gemcitabine and oridonin. These results suggested oridonin may enhance the chemosensitivity of PANC-1/Gem cells to gemcitabine by downregulating the expression of MRP1 and GST pi.

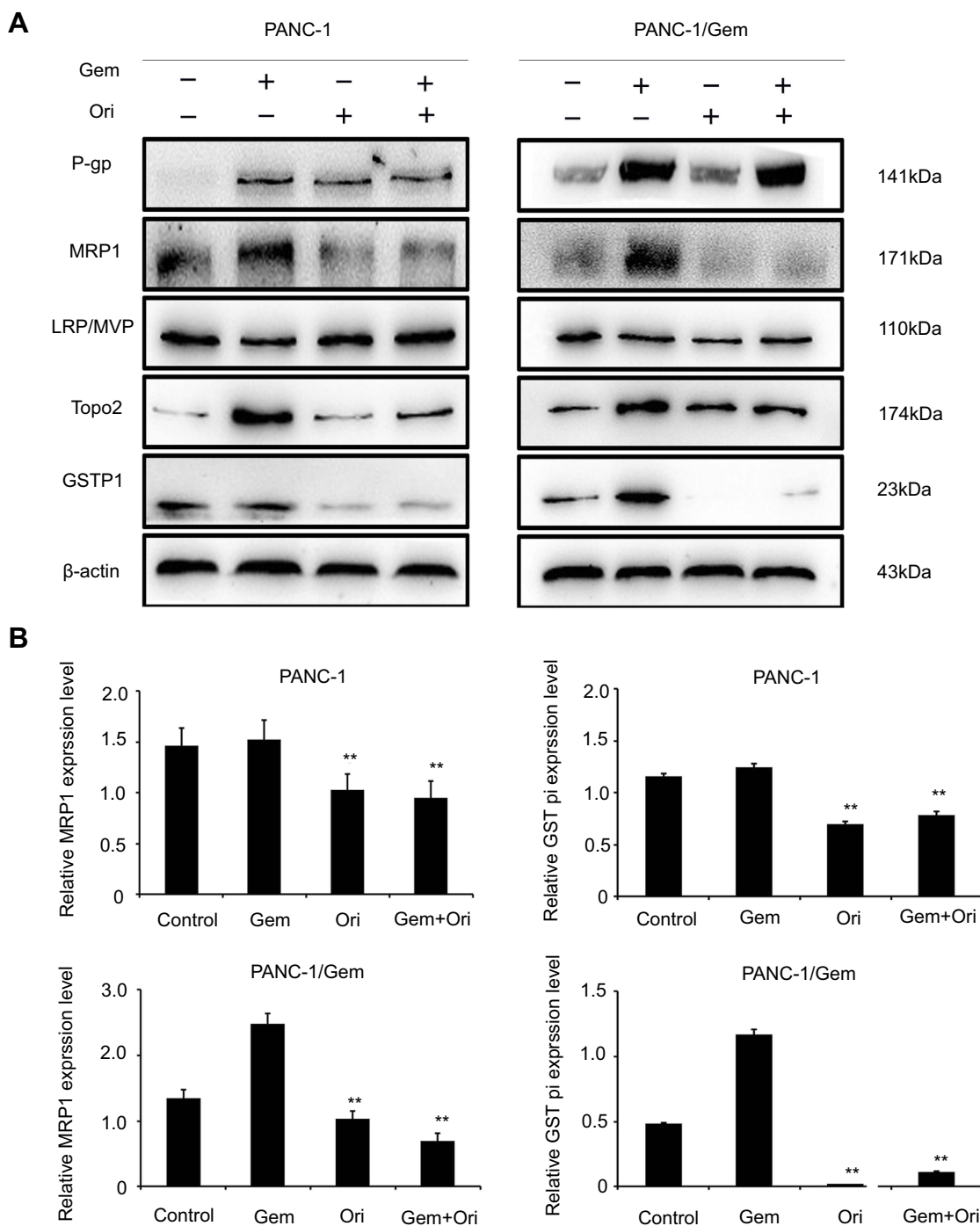


Figure 2 Oridonin regulates the expression of multidrug resistance proteins in PANC-1/Gem and PANC-1 cells. **(A)** Western blot analysis of P-gp, MRP1, LRP/MVP, Topo2 and GST pi protein expression in PANC-1 and PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h. β-actin was used as a loading control. **(B)** Expression of MRP1 and GST pi protein in PANC-1 and PANC-1/Gem cells were detected by Western Blot analysis. Data were expressed as the mean ± standard deviation. **P<0.01. **Abbreviations:** P-gp, P glycoprotein; MRP1, multidrug resistance protein 1; LRP/MVP, lung resistance protein/major vault protein; GST, glutathione S-transferase.

Oridoninovercomes PANC-1/Gem cell gemcitabine resistance by suppressing GST pi level

To further examine whether oridonin overcomes gemcitabine resistance by suppressing GST pi expression

in gemcitabine-resistant cells, GST pi was observed in oridonin-treated and gemcitabine-treated PANC-1/Gem cells using immunofluorescence assay. The immunofluorescence result was consistent with Western blot analysis. Compared with the control group, the expression of GST

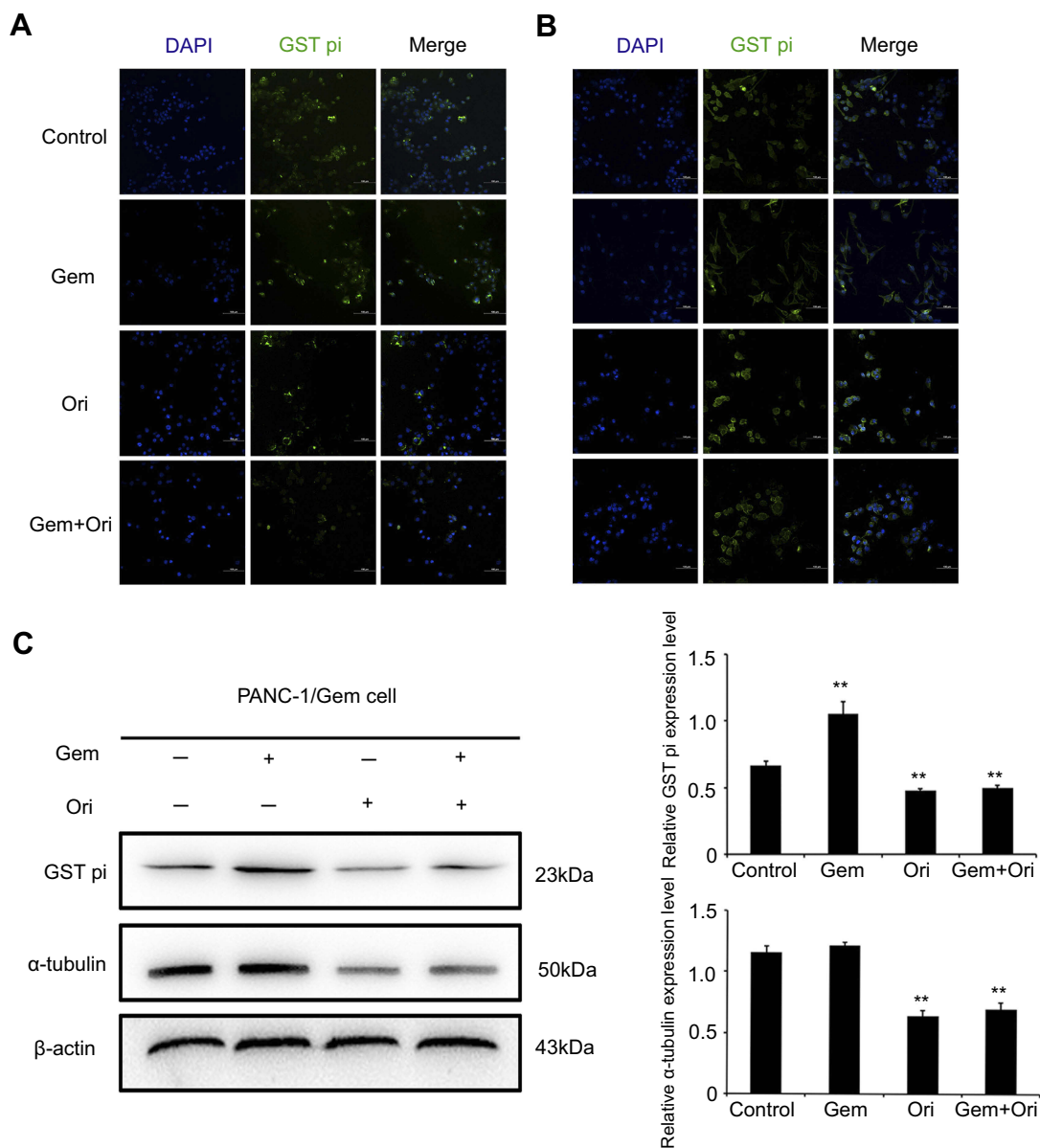


Figure 3 Oridonin overcomes PANC-1/Gem cell gemcitabine resistance by suppressing GST pi expression and changing the structure of cytoskeleton. **(A and B)** Immunofluorescence staining for GST pi and α -tubulin in PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h (magnification, x200). **(C and D)** Western blot analysis of GST pi and α -tubulin in PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h. β -actin was used as a loading control. Data were expressed as the mean \pm standard deviation. ** $P < 0.01$.

Abbreviation: GST, glutathione S-transferase.

pi protein was decreased in PANC-1/Gem cells treated with oridonin. Notably, gemcitabine treatment upregulated the expression levels of GST pi, whereas the combination of gemcitabine and oridonin downregulated GST pi expression (Figure 3A). These results suggested that oridonin overcomes PANC-1/Gem cell gemcitabine resistance by suppressing the expression of GST pi.

Oridonin overcomes PANC-1/Gem cell gemcitabine resistance by modifying the structure of the cytoskeleton

α -tubulin is a primary component of the cytoskeleton. Previous studies have reported that α -tubulin has an important role on tumor cell proliferation, progression and

migration.¹² As indicated in **Figure 3B**, the expression of α -tubulin in PANC-1/Gem cells was significantly changed following treatment with oridonin and gemcitabine. PANC-1/Gem cells alone were predominantly fusiform. However, the number of cells that were fusiform was increased following gemcitabine treatment. Notably, PANC-1/Gem cells treated with oridonin became deformed and round in shape. Western blot analysis revealed that the expression of α -tubulin was decreased in cells treated with oridonin (**Figure 3C**). These results suggested that oridonin may overcome PANC-1/Gem cell gemcitabine resistance by suppressing GST pi expression and modifying the structure of the cytoskeleton.

Oridonin overcomes PANC-1/Gem cell gemcitabine resistance by inducing cell apoptosis

The effects of oridonin and gemcitabine on PANC-1/Gem cell apoptosis were assessed by flow cytometry. Cells were treated with drugs at their IC_{50} value for 24 h. The results indicated that oridonin had a greater significant apoptosis-inducing effect compared with gemcitabine. However, the combination of gemcitabine and oridonin could achieve a similar effect (**Figure 4A and B**). The Caspase and Bcl-2 family of proteins serve important roles in apoptotic cell death. The protein expression levels of Caspase 3, Caspase 9, Bax and Bcl-2 were investigated using Western blot analysis. After treatment with gemcitabine, oridonin, combined of gemcitabine and oridonin; Caspase 3, Caspase 9 and Bax expression levels were significantly increased compared with control. By contrast, Bcl-2 expression was significantly downregulated after 24 h of treatment with oridonin or combined of gemcitabine and oridonin (**Figure 4C and D**). Above results indicated that oridonin effectively induced PANC-1/Gem cell apoptosis and increased the sensitivity to gemcitabine.

Oridonin overcomes PANC-1/Gem cell gemcitabine resistance by suppressing tumorigenicity in nude mice

It was determined whether oridonin in combination with gemcitabine could inhibit PANC-1/Gem cell tumorigenicity in mouse models (**Figure 5C**). Nude mice were subcutaneously injected with PANC-1/Gem cells and randomly assigned into four groups. Treatment with gemcitabine, oridonin, both agents and control began 1 week after injection and lasted for 7 weeks (**Figure 5A**). Normal pancreatic tissues and transplanted tumors were stained

with H&E. Notably, the surface of normal pancreatic tissues was covered with connective tissue. The connective tissue extended into the gland and was divided into various leaflets. The interlobular connective tissue contained blood vessels, ducts and lymphatic vessels. However, in heteroplastic PANC-1/Gem cells gland lobules were not observed, the pancreatic cells were irregular and loose, there was no glandular cavity or clear lumen and the nuclei were irregular in shape (**Figure 5B**).

Compared with the control group, tumors treated with gemcitabine or oridonin alone had a similar histopathological effect. However, combination treatment with oridonin and gemcitabine decreased tumor growth (**Figure 5C–E**). Immunohistochemical staining indicated that oridonin treatment caused a decrease in the expression levels of GST pi in the transplanted tumor tissue compared with control (**Figure 5F**). These results revealed that oridonin could overcome PANC-1/Gem cell gemcitabine resistance by suppressing tumorigenicity.

Oridonin overcomes gemcitabine resistance in PANC-1/Gem cells by knockdown of LRP1

Previous work has demonstrated that metabolic alterations can regulate response to gemcitabine in pancreatic cancer.^{13,14} Therefore, ATP levels in pancreatic cancer cells were assessed following various treatments. The results suggested the ATP levels were increased in PANC-1/Gem cells when compared with PANC-1 cells. However, ATP levels were not significantly different after gemcitabine treatment in PANC-1/Gem cells compared with PANC-1/Gem cells. Notably, following treatment with oridonin or combined of gemcitabine and oridonin, ATP levels were significantly decreased compared with PANC-1/Gem cells (**Figure 6A**).

Lipid metabolism is an important factor of energy metabolism. The present study identified that LRP1 was highly expressed in PANC-1/Gem cells. Western blot analysis demonstrated that oridonin treatment resulted in decreased LRP1 expression, which suggests it may have an important role in gemcitabine resistance (**Figure 6B**). Furthermore, immunofluorescence results were consistent with Western blot analysis (**Figure 6C**). To investigate our hypothesis that LRP1 may be a novel target in gemcitabine resistance, LRP1 expression was knocked down through lentiviral vector-mediated RNA silencing in the PANC-1/Gem cells with shLRP1, shNC was considered the control.

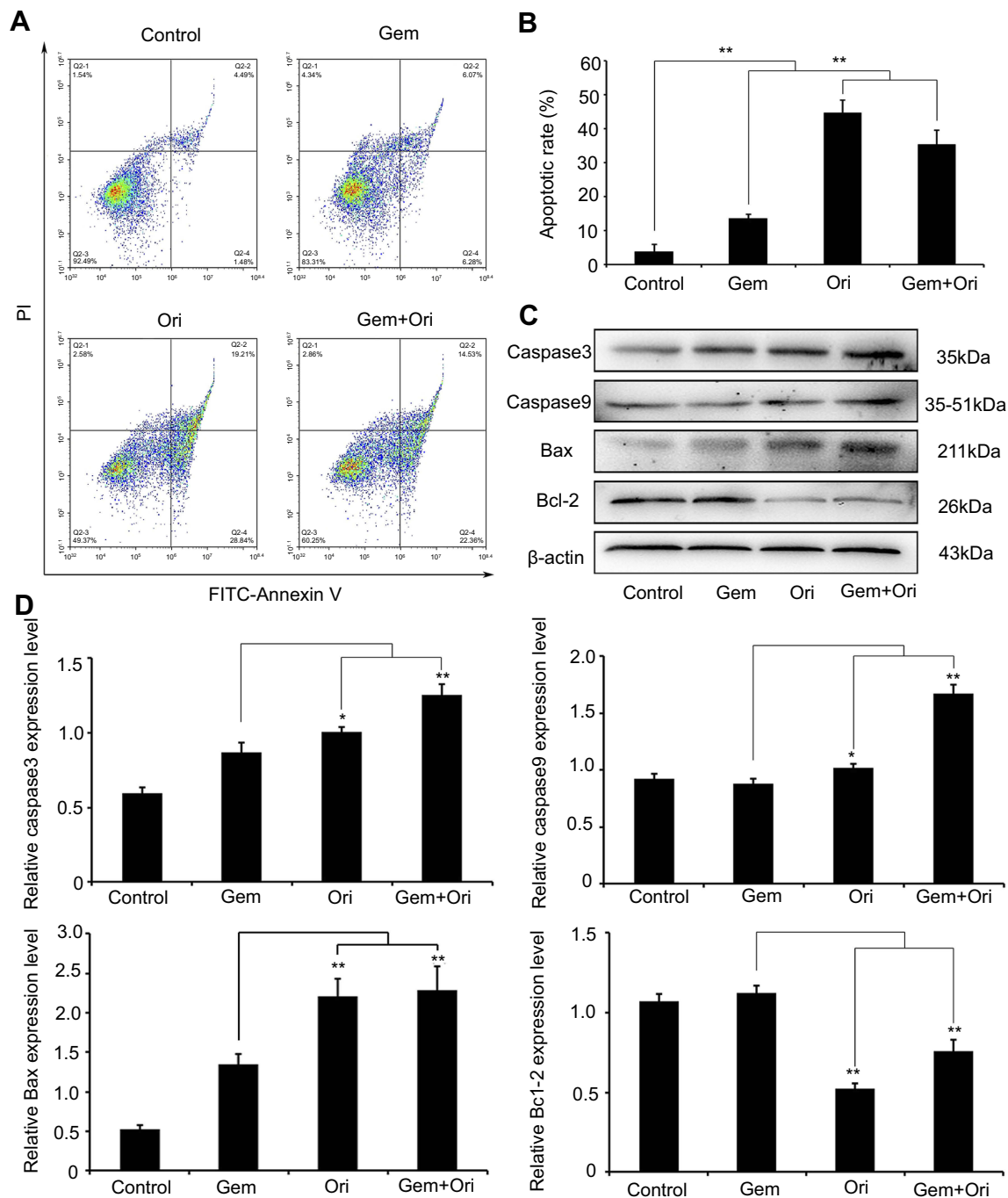


Figure 4 Oridonin overcomes PANC-1/Gem cell gemcitabine resistance by inducing cell death. (A and B) PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h were analyzed using flow cytometry. Data were expressed as the mean \pm standard deviation. $**P<0.01$. (C) Western blot analysis of Caspase 3, Caspase 9, Bax or Bcl-2 protein expression in PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h. β -actin was used as a loading control. (D) Expression of Caspase 3, Caspase 9, Bax or Bcl-2 protein in PANC-1/Gem cells was detected. $*P<0.05$ and $**P<0.01$. **Abbreviations:** Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated x protein.

It was observed that endogenous LRP1 protein expression levels in shLRP1 cells were decreased compared with shNC cells (Figure 7A). Furthermore, the effect of gemcitabine on cell viability was also assessed. shLRP1 cells became more sensitive to gemcitabine when compared

with shNC cells (Figure 6D). The colony formation assays demonstrated that LRP1 knockdown effectively suppressed cell growth and cell proliferation (Figure 6E).

Notably, it has been reported that LRP1 is a primary regulator of ERK and JNK signaling in tumor cells.¹⁵

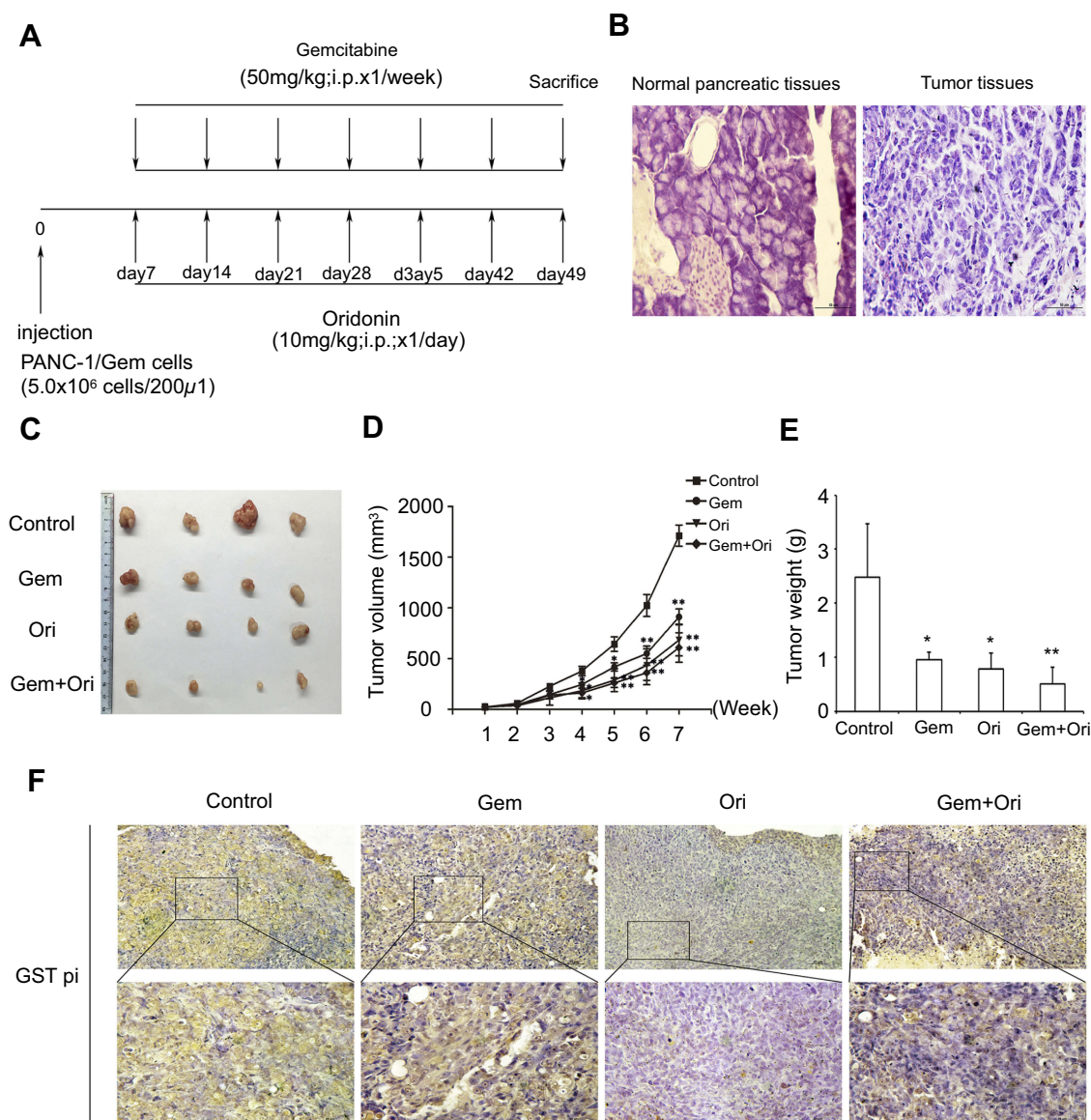


Figure 5 Oridonin overcomes PANC-1/Gem cell gemcitabine resistance in vivo. **(A)** Tumor progression timeline with experimental treatment time points. **(B)** Normal pancreatic tissues and transplantation of PANC-1/Gem cell tumor stained with hematoxylin and eosin. **(C)** Representative images of tumors formed in nude mice. **(D)** Statistical comparisons of tumor volumes were measured on the indicated days. Data were expressed as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$. **(E)** Weight of the transplanted tumor. * $P < 0.05$ and ** $P < 0.01$. **(F)** Xenografts from mice were immunochemically stained using GST pi antibodies.

Abbreviation: GST, glutathione S-transferase.

Results in the present study indicated that ERK-1/2 protein expression levels were selectively decreased and JNK-1/2/3 protein expression levels were increased in LRP1-silenced cells. Oridonin treatment resulted in the same effects as shLRP1 cells. NF- κ B activity serves an important role in gemcitabine resistance.¹⁶ The present study indicated that silencing LRP1 expression could suppress NF- κ B activity. PANC-1/Gem cells treated with oridonin or combined of gemcitabine and oridonin also inhibited NF- κ B expression compared with control (Figure 7A). In

addition, immunohistochemical staining in tumor tissues revealed that oridonin treatment decreased the expression of LRP1 (Figure 7B). These results suggest that oridonin may overcome PANC-1/Gem cell gemcitabine resistance by regulating LRP1 expression.

Discussion

Chemotherapy has become the primary treatment for advanced pancreatic cancer in the clinic. Although gemcitabine-based chemotherapy has improved the survival of

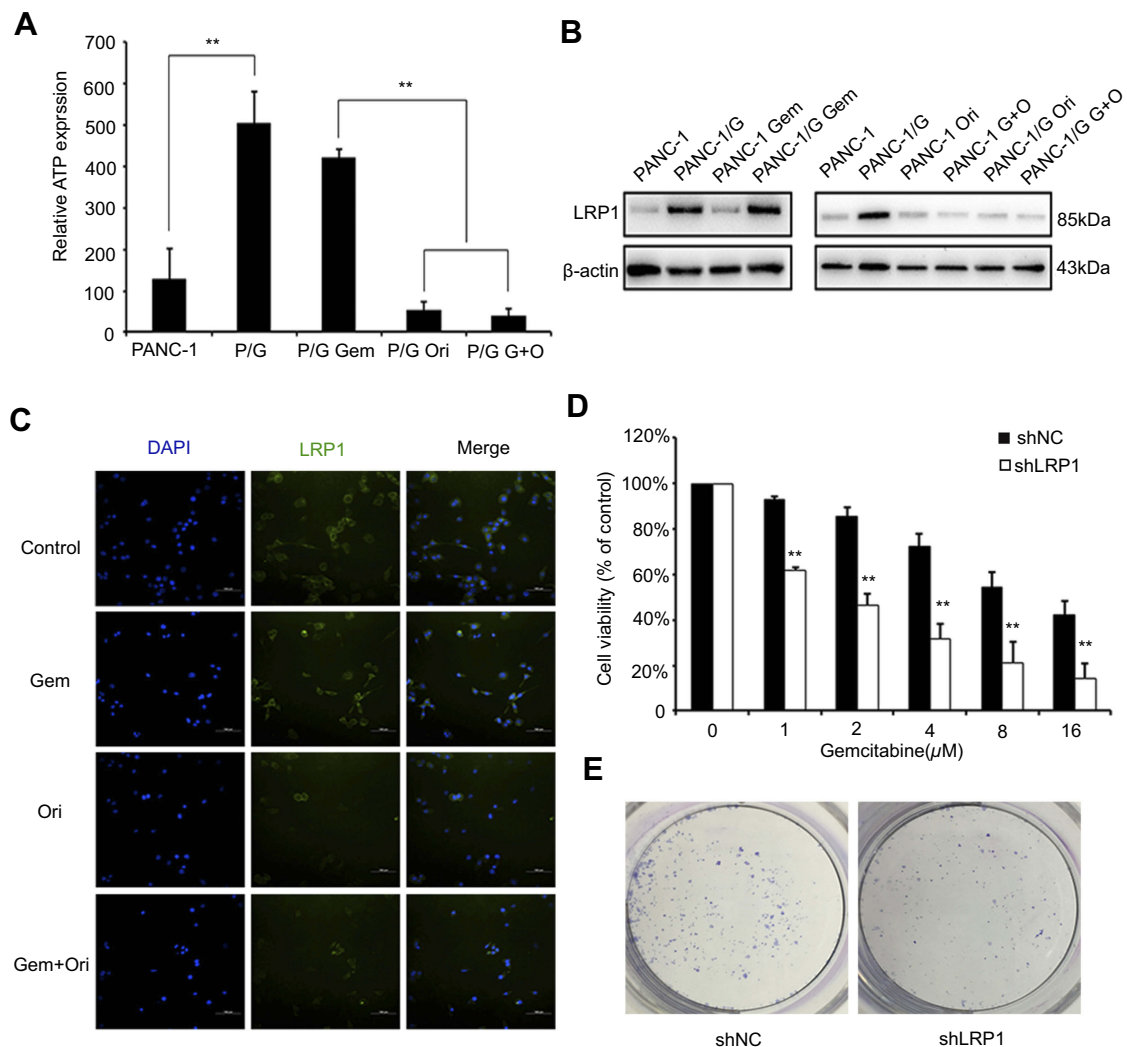


Figure 6 Oridonin enhances the sensitivity of PANC-1/Gem cells to gemcitabine and inhibit cell growth after LRP1 silencing. **(A)** ATP levels were examined under different treatment in PANC-1/Gem and PANC-1 cells for 24 h. **(B)** Western blot analysis of LRP1 expression in PANC-1 and PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h. β -actin was used as a loading control. **(C)** Immunofluorescence staining for the LRP1 in PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h (magnification, $\times 200$). **(D)** Cell viability detected by Cell Counting Kit-8 assays in shNC- and shLRP1-treated PANC-1/Gem cells that received different doses of gemcitabine for 24 h. $**P < 0.01$. **(E)** Images of the shNC and shLRP1 cells were analyzed in a clonogenic assay.

Abbreviations: LRP1, low-density lipoprotein receptor protein 1; shLRP1, short hairpin LRP1; shNC, non-silencing control shRNA (shNC).

patients with pancreatic cancer, the effect achieved is still limited. The failure of chemotherapy is due to multiple factors, including the extrinsic or intrinsic resistance to conventional therapies.^{17,18} Therefore, improving the sensitivity of cancer cells to chemotherapeutic agents could increase the survival of patients. In the present study, a highly gemcitabine-resistant pancreatic cancer cell line, PANC-1/Gem, was established, which stemmed from parental PANC-1 cell lines. It was indicated that the growth of PANC-1/Gem cells was significantly inhibited after treatment with oridonin. However, there was no significant difference compared with PANC-1 cells after treatment with oridonin (Figure 1B). Additionally, oridonin and

gemcitabine were indicated to have a synergistic effect on PANC-1/Gem cells (Figure 1D), which was also suggested in xenograft experiments (Figure 5). These results indicate that oridonin could effectively overcome gemcitabine resistance in pancreatic cancer cells.

The mechanism of drug resistance in pancreatic cancer is complex. The drug efflux pump and drug detoxification enzyme system serve an important role in drug resistance. ATP-binding cassette (ABC) membrane-transport proteins, including P-gp, MRP and LRP/MVP, are associated with decreasing intracellular accumulation of various chemotherapeutic agents. Previous studies have reported that P-gp, MRP and LRP/MVP are overexpressed in pancreatic

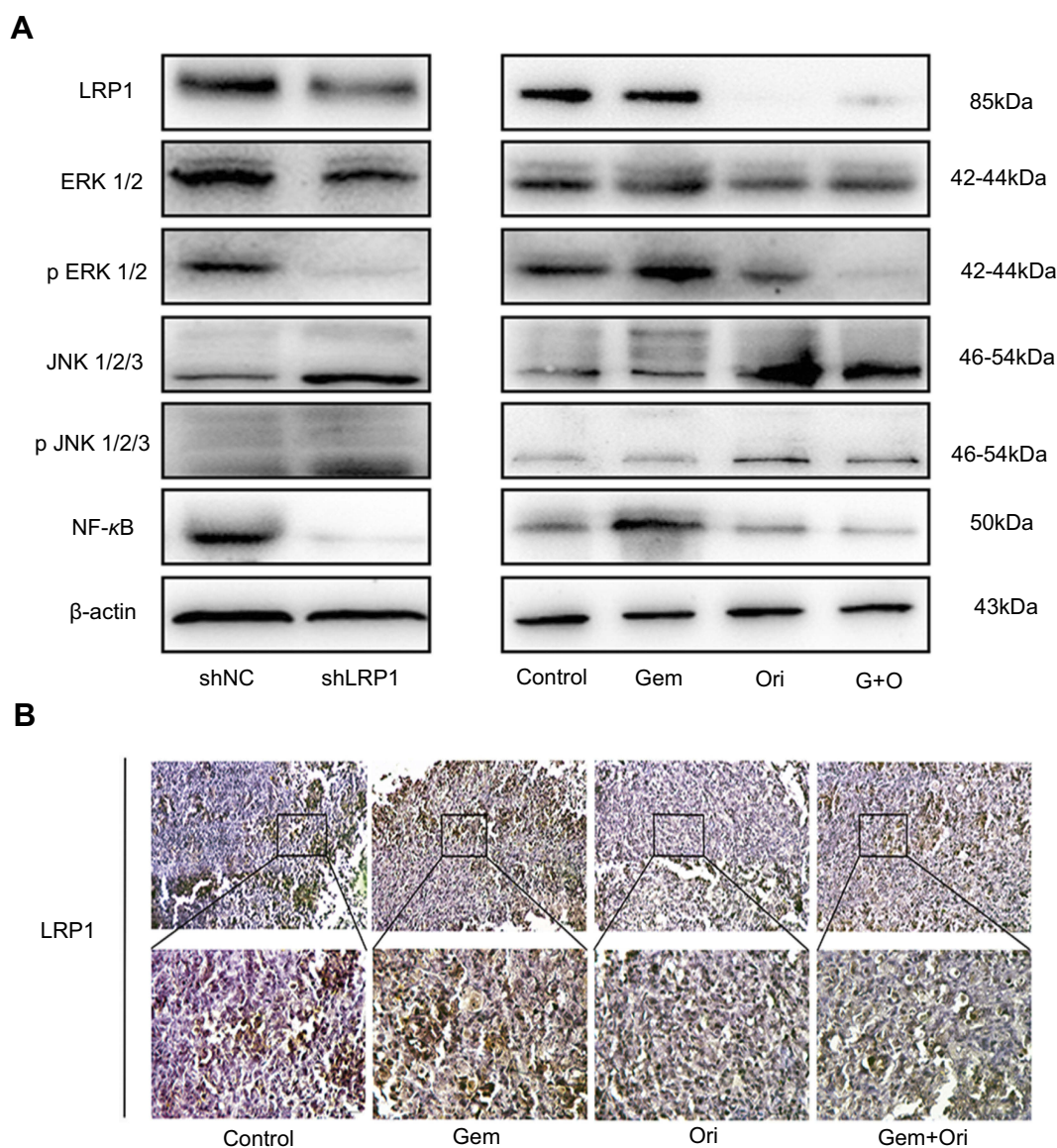


Figure 7 Oridonin overcomes PANC-1/Gem cell gemcitabine resistance by regulating LRP1/ERK/JNK signaling. **(A)** Expression of LRP1, ERK1/2, phosphorylated-ERK1/2, JNK1/2/3, phosphorylated-JNK, and NF-κB were examined in shNC and shLRP1 cells. The same proteins were detected in PANC-1/Gem cells treated with gemcitabine, oridonin or both for 24 h. **(B)** Xenografts from mice were immunochemically stained using LRP1 antibodies.

Abbreviations: LRP1, low-density lipoprotein receptor protein 1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF, nuclear factor; shLRP1, short hairpin LRP1; shNC, non-silencing control shRNA (shNC).

cancer.^{19–21} GST pi, which is a member of the GST family, has been demonstrated to have an important role in tumor multidrug resistance. Through catalyzing the combination of glutathione with electrophilic agents, intracellular drug accumulation can be decreased. The overexpression of GST pi has been reported in various types of cancer, including breast cancer, colon cancer, kidney cancer, lung cancer and ovarian cancer.^{22–24} Multiple studies have demonstrated that the expression of GST pi in pancreatic cancer is significantly higher than that in normal tissues, and the sensitivity of pancreatic cancer to chemotherapeutic agents could

be restored by inhibiting the expression of GST pi.²⁵ However, there are few reports regarding the role of ABC membrane-transport protein and GST in mediating cancer cells that are resistance to gemcitabine. In the present study, it was identified that P-gp, MRP1 and GST pi proteins were overexpressed in PANC-1 cells compared with control. Similarly, increased expression of P-gp, MRP1 and GST pi was indicated after treatment with gemcitabine compared with control (Figure 2A). The results indicated that these proteins may be associated with gemcitabine-resistance. However, the expression of MRP1 and GST pi proteins

was downregulated after treatment with oridonin compared with control. Following combination treatment of gemcitabine and oridonin, the overexpression of these proteins was reduced (Figure 2A and B). Decreased expression of GST pi protein was detected in transplanted tumor tissue following oridonin treatment (Figure 5F). The present study illustrates that GST pi may serve an important role in the development of gemcitabine resistance in pancreatic cancer.

Studies have suggested that the abnormal expression and distribution of tubulin is associated with tumorigenesis, cell proliferation and malignant biological behavior of tumor cells.²⁶ Banerjee et al reported that the increase of α -tubulin expression is significantly associated with paclitaxel drug resistance.²⁷ Therefore, inhibition of tubulin expression could restore the sensitivity of tumor cell. In our previous study, oridonin could significantly change the cytoskeleton of PANC-1 cells and downregulate α -tubulin expression, which may be associated with the inhibition of migration in pancreatic cancer.

Cell apoptosis is an important factor during the development of drug resistance. The Bcl-2 family has been demonstrated to have an important role in cell apoptosis. The downregulation of Bcl-2 and the upregulation of Bax triggers the activity of Caspase 3 and Caspase 9, resulting in cell apoptosis. Studies have indicated that the overexpression of Bcl-2 is associated with gemcitabine resistance.²⁸ Notably, the Bcl-2 family is implicated in the intrinsic gemcitabine resistance of pancreatic cancer.²⁹ After inhibiting the expression of Bcl-2 and increasing cell apoptosis may reverse the resistance of gemcitabine.³⁰ Zhang et al found that trichosanthin (TCS), an antiviral plant defense protein found in the root tuber of a TCM herb, induces apoptosis through the Bcl-PARP signaling pathway.³¹ The study revealed that gemcitabine could increase apoptosis in PANC-1/Gem cells. Furthermore, oridonin could inhibit the growth of PANC-1/Gem cells and also increased cell apoptosis. Results from the combination group suggested that oridonin could overcome the resistance of gemcitabine by inducing cell apoptosis (Figure 4A and B). Notably, it was demonstrated that oridonin could decrease the expression of Bcl-2, but increase the expression of Bax, Caspase 3 and Caspase 9 (Figure 4C and D). Therefore, oridonin could enhance the sensitivity of gemcitabine-resistant pancreatic cells.

Accumulating evidence suggests that metabolic alterations serve an important role in cancer development and progression. Energy metabolism in tumor cells is extremely active, particularly in malignant tumor cells. Zhuo et al reported that oridonin can inhibit the glucose

metabolism in colorectal cancer cells and influence the cellular ATP supply.³² The present results demonstrated that ATP activity was significantly increased in PANC-1/Gem cells compared with the normal cells. After 24 h of treatment with oridonin, ATP levels decreased (Figure 6A). With the depletion of ATP, tumor cells were more sensitive to chemotherapy in a previous study.³³ Moreover, recent research has indicated that pancreatic cancer is highly dependent on cholesterol. Via LRP, cholesterol and low-density lipoprotein are absorbed and can accumulate in pancreatic cancer cells. It has been observed that LRP is overexpressed in pancreatic cancer cells.³⁴ However, silencing of LRP expression can reduce cholesterol uptake and tumor cell proliferation. In addition, with the blockade of cholesterol uptake, drug sensitivity of pancreatic cancer to chemotherapeutic drugs increases.³⁵ Based on these findings, it was suggested combinative treatment with oridonin and gemcitabine may overcome the multidrug resistance. Notably, the present study indicated LRP1 was highly expressed in gemcitabine-resistant PANC-1/Gem cells. However, oridonin could downregulate LRP1 expression (Figure 6B and C). Furthermore, the present results demonstrated that oridonin could enhance the sensitivity of PANC-1/Gem cells to gemcitabine and inhibit cell growth after LRP1 silencing (Figure 6D and E).

JNK is a stress-activated protein kinase, which is one of the major members of the mitogen-activated protein kinase (MAPK) family. It is involved in cellular physiology and pathological processes. The unbalanced function of JNK leads to various diseases, including tumors, inflammation and ischemia-reperfusion injury.³⁶ In malignant tumors, activated JNK signaling pathways are associated with enhanced resistance to anti-tumor agents and inhibition of apoptosis.^{37,38} In addition, ERK is an important member in the MAPK family. The ERK signaling pathway can transmit extracellular stimuli for the nucleus and regulates tumorigenesis, proliferation, apoptosis and drug resistance.^{39,40} Benoit et al demonstrated that LRP1 promotes tumor cell proliferation and invasion by upregulating ERK and inhibiting the JNK signaling pathway.⁴¹ The study suggests that the growth of tumor cells could regulate downstream JNK and ERK signaling by LRP1 gene knockdown. The present results indicated that LRP1 protein expression was significantly decreased in PANC-1/Gem cells following LRP1 knockdown when compared with the shNC group. After LRP1 knockdown, the expression of phosphorylated ERK was decreased. However, the

expression of phosphorylated JNK was increased in PANC-1/Gem cells after LRP1 knockdown (Figure 7A). Notably, oridonin could downregulate the expression levels of ERK and phosphorylated ERK. However, the expression levels of JNK and phosphorylated JNK were upregulated in PANC-1/Gem cells. The results revealed that oridonin inhibits cell proliferation and promotes apoptosis by regulating JNK and ERK signaling proteins in PANC-1/Gem cells.

NF- κ B is an important member in the transcriptional regulators family. Activated NF- κ B may regulate the transcription of genes involved in the occurrence of various diseases and has an important role in cell differentiation, apoptosis and resistance in malignant tumors.⁴² Various studies have indicated that gemcitabine treatment could increase NF- κ B in pancreatic cancer cells and reduce the anti-tumor effect. However, inhibiting the activation of NF- κ B could reverse the resistance of pancreatic cancer to gemcitabine.⁴³ The present results indicated the expression of NF- κ B was significantly increased in PANC-1/Gem cells after treatment with gemcitabine. Furthermore, the expression of NF- κ B in PANC-1/Gem cells was significantly inhibited by oridonin and combination treatment (Figure 7A). Knockdown of LRP1 gene also reduced NF- κ B in PANC-1/Gem cells. However, the relevant mechanisms require further investigation.

In conclusion, the present study indicated that oridonin could overcome gemcitabine resistance in PANC-1/Gem cells by regulating GST pi and LRP1/ERK/JNK signaling, inducing cell apoptosis. Therefore, oridonin with gemcitabine may be a promising preoperative treatment for patients who suffer from pancreatic cancer.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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