

Harmine suppresses the proliferation of pancreatic cancer cells and sensitizes pancreatic cancer to gemcitabine treatment

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Purpose: Pancreatic carcinoma is one of the most deadliest types of cancer, and relatively insensitive to the currently available chemotherapy. Thus, the discovery of novel therapeutic agents to prolong the survival times of patients with pancreatic cancer is urgently required.

Methods: Cell proliferation was assessed using the sulforhodamine B and cell clone formation assay, apoptosis was analyzed through Annexin V/PI staining, analysis of cell cycle distribution was determined by PI staining, and the expression of proteins was detected via Western blotting.

Results: Our data showed that harmine exerted an anti-proliferative effect and cell cycle arrest at G2/M in pancreatic cancer cells. Meanwhile, harmine plus gemcitabine showed strong synergy in inhibiting the proliferation of pancreatic cancer cells. Furthermore, harmine induced apoptosis and enhanced the gemcitabine-induced apoptosis in pancreatic cancer cells. The AKT/mTOR pathway is involved in mechanisms of gemcitabine resistance in pancreatic cancer cells, our data demonstrated that harmine plus gemcitabine significantly suppressed the AKT/mTOR signaling pathway.

Conclusion: Harmine may be a potential candidate for the treatment of pancreatic cancer. Moreover, the combination of harmine with gemcitabine appears to be an attractive option for the treatment of patients with pancreatic cancer.

Keywords: harmine, gemcitabine, pancreatic cancer, AKT/mTOR, combination

Introduction

Pancreatic carcinoma is one of the most deadliest types of cancer worldwide. In addition, it is estimated that pancreatic carcinoma will become the second leading cause of cancer-related death by 2030.¹ During the previous several decades, there has not been significant improvement in the clinical outcome of pancreatic cancer, with the average survival rate being 5%.² The anatomical location of the pancreatic gland is close to many important organs, main arteries and veins. Consequently, a large proportion of patients with pancreatic carcinoma (80–85%) present with an extension to adjacent organs or distant metastases, rendering surgical resection.³ Currently, gemcitabine is recommended as the first-line drug for the treatment of advanced pancreatic cancer.⁴ However, pancreatic cancer is relatively insensitive to chemotherapy, and the poor outcomes associated with chemotherapy is largely related to acquired chemoresistance of pancreatic cancer cells.⁵ Thus, the discovery of novel agents or gemcitabine-based combination regimens are urgently required to overcome chemoresistance and prolong the survival time of patients with pancreatic cancer.

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Harmine is a tricyclic β -carboline alkaloid isolated from the seeds of the medical plant *Peganum harmala* L.⁶ The plant is the only salt-tolerant perennial herb in the *Peganum* genus of the family Zygophyllaceae. Its seeds are traditionally used to relieve pain, to promote blood circulation, and to treat rheumatism and symptoms (ie, cough and asthma).⁷ Furthermore, harmine can also inhibit all members of the DYRK family (ie, DYRK1A, DYRK1B, DYRK2, and DYRK4), with the highest affinity demonstrated for DYRK1A.⁸ In addition, several molecules, such as AChE, BChE, MAO-A and GABA receptor, were also considered to be targets of harmine for other pharmacological effects.⁹ Harmine possesses multiple types of pharmacological activity such as anti-microbial, anti-fungal, anti-tumor, anti-plasmodial, anti-oxidant, anti-mutagenic, and anti-genotoxic properties.¹⁰ Harmine suppresses the proliferation of various types of cancer cells (eg, ovarian and gastric cancer).^{11,12} It has been shown to exhibit anti-cancer properties by inhibiting tumor growth, metastasis, and inducing apoptosis.¹³ Our data demonstrated for the first time that harmine may exhibit anti-cancer activity in pancreatic cancer cells. In addition, harmine may sensitize pancreatic cancer cells to treatment with gemcitabine via AKT/mTOR pathway. Our study has revealed that harmine may be a potential candidate for the treatment of pancreatic cancer treatment, and the combination of harmine with gemcitabine appears to be an attractive option for the treatment of patients with pancreatic cancer.

Materials and methods

Materials

Harmine (cat. no. HY-N0737A) and gemcitabine hydrochloride (cat. no. HY-B0003) were supplied by Med Chem Express (Monmouth Junction, NJ, USA).

Cell culture

PANC-1 (cat. no. TCHu98), CFPAC-1 (cat. no. TCHu112), SW-1990 (cat. no. TCHu201) and BxPC-3 (cat. no. TCHu12) were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). BxPC-3, CFPAC-1 and SW1990 cells were maintained under humidified conditions (37 °C, 5% CO₂) in RPMI-1640 (Life Technologies) plus 5% FBS. PANC-1 cells were cultured in DMEM medium.

Sulforhodamine B (SRB) assay

Cell survival was determined by SRB assay as described previously.¹⁴ Firstly, pancreatic cancer cells were seeded

into 96-well plates at a density of 4×10^3 per well and treated with the compounds at the indicated concentrations for 72 h. Then, the cells were fixed by 10% trichloroacetic acid solution at 4 °C for 1 h, washed with tap water, dried and stained with 0.4% SRB solution for 30 min. Then, the excess dye was removed by washing repeatedly with 1% acetic acid. Following this, wells were dried and SRB dye was dissolved in 10 mM Tris base solution for OD determination at 515 nm using a multi-scan spectrum.

Cell clone formation assay

Pancreatic cancer cells were seeded onto 6-well plates at a density of 1,000 cells per well and treated with harmine at the indicated concentrations, plated in 35 mm dishes and incubated at 37 °C for 14 days. The compound-containing medium was replaced every 2–3 days during the whole experiment.

Apoptosis detection by annexin v/propidium iodide (PI) staining

Apoptosis was detected by Annexin V - FITC apoptosis detection kit (cat. no. 556547, BD bioscience, San Jose, CA) according to the manufacturer's recommendations. Briefly, cells were collected and incubated in 200 μ L binding buffer containing 10 μ L Annexin V at room temperature for 10 mins. Prior to flow cytometric analysis, 5 μ L propidium iodide solution was added.

Analysis cell cycle distribution by PI staining

Cell cycle distribution was detected by PI staining as described previously.¹⁵ Samples were analyzed by a FACSCalibur cytometer.

Western blotting

Whole-cell extracts for SDS-PAGE were prepared and Western blotting analysis was performed as previously described.¹⁴ The antibodies used for Western blotting were obtained from different resources: anti-c-myc antibody (cat. no. 13987), anti-cleaved PARP antibody (cat. no. 9541), anti-cleaved-caspase-3 antibody (cat. no. 9661), anti-caspase-3 antibody (cat. no. 9662), anti-AKT antibody (cat. no. 9272), anti-phospho-AKT (Ser-473) antibody (cat. no. 4051), anti-phospho-p70S6 Kinase (Thr-389) antibody (cat. no. 9234), anti-mTOR antibody (cat. no. 2983), anti-p70S6 Kinase antibody (cat. no. 2708) and anti-phospho-mTOR (Ser-2448)

antibody (cat. no. 5536) were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-p21 antibody (cat. no. sc-6246) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA); anti-GAPDH antibody (cat. no. db106) was acquired from Diagbio Biosciences (Hangzhou, China); anti-cyclin B1 antibody (cat. no. 55004-1-AP) was purchased from Proteintech (Rosemont, IL, USA).

Statistical analyses

The data were presented as means \pm SD from at least three independent experiments. Differences between the groups were determined with Student's *t*-test analysis and considered significant when $P < 0.05$. Combination index (CI) values were calculated using Calcsyn (Biosoft, Great Shelford, Cambridge, UK) and it indicated synergism when CI value < 0.9 .

Results

Harmine suppresses the proliferation of pancreatic cancer cells

Harmine shows anti-cancer activity in multiple types of cancer, such as neuroblastoma, ovarian, and gastric cancer. However, thus far, its activity against pancreatic cancer has not been evaluated.^{11,12,16} Firstly, we determined the anti-cancer effect of harmine on the growth of pancreatic cancer cell lines, including PANC-1, CFPAC-1, SW-1990, and BxPC-3. Pancreatic cells were incubated with indicated concentrations of harmine for 24 h, 48 h, and 72 h. As shown in [Figure 1A](#), the growth of pancreatic cancer cells was significantly suppressed by harmine in a dose- and time-dependent manner. The half-maximal inhibitory concentrations (IC₅₀) of harmine were shown in [Figure 1B](#). In addition, harmine significantly suppressed the colon formation of pancreatic cells in a dose-dependent manner ([Figure 1C](#)). Moreover, we observed that harmine induced cell cycle arrest at G₂/M in PANC-1 and BxPC-3 cells ([Figure 1D](#)). Meanwhile, harmine-induced G₂/M arrest was accompanied by the activation of p21 and cyclin B1, as well as inhibition of c-Myc in pancreatic cancer cells ([Figure 1E](#)). Thus, our data demonstrated that harmine may exert an anti-proliferative effect and cell cycle arrest at G₂/M in pancreatic cancer cells.

Harmine enhanced the cytotoxicity of gemcitabine in pancreatic cancer cells

Pancreatic cancer is typically diagnosed at an advanced stage and gemcitabine-based treatment has been used as

first-line therapy for metastatic pancreatic cancer.¹⁷ Thus, we are encouraged to investigate whether harmine may enhance the cytotoxicity of gemcitabine in pancreatic cancer cells. As shown in [Figure 2A](#), we determined its anti-cancer activity by combining harmine with gemcitabine at clinically achievable concentrations in four human pancreatic cancer cell lines.¹⁸ The combination of harmine plus gemcitabine showed strong synergy in these pancreatic cancer cell lines (mean CI values: < 0.9 , [Figure 2B](#)).

Harmine enhanced the gemcitabine induced apoptosis in pancreatic cancer cells

Initially, we investigated the morphological changes induced by the combination of harmine and gemcitabine in BxPC-3 and PANC-1 cells. [Figure 3A](#) showed that a large proportion of pancreatic cancer cells treated with harmine plus gemcitabine were detached from the culture dishes, whereas the remaining adherent cells showed loss of adhesion, shrinkage, and rounding, which are typical morphological changes associated with apoptosis. Subsequently, we investigated the apoptotic effect of harmine, gemcitabine, and harmine plus gemcitabine in PANC-1 and BxPC-3 cells. As shown in [Figure 3B](#), according to Annexin V positivity, the percentages of apoptotic PANC-1 cells were as follows: vehicle control, 7.33% \pm 0.18%; gemcitabine, 35.60% \pm 1.56% (harmine vs harmine plus gemcitabine, $P = 0.0032$); harmine, 19.75% \pm 1.34% (gemcitabine vs harmine plus gemcitabine, $P = 0.0015$); and harmine plus gemcitabine, 74.63% \pm 2.72%. Harmine enhanced gemcitabine induced apoptosis by in both PANC-1 and BxPC-3 cells ([Figure 3B](#)). We also found that the cleavage of PARP and activation of caspase-3 were enhanced following treatment with harmine plus gemcitabine versus either single-agent treatment in pancreatic cancer cells ([Figure 3C](#)). Thus, we concluded that harmine induces apoptosis and enhances gemcitabine induced apoptosis in pancreatic cancer cells.

Harmine plus gemcitabine inhibited the AKT/mTOR signaling pathway

The PI3K/AKT/mTOR pathway is involved in the development of resistance to gemcitabine. Our data indicated that harmine suppressed the AKT/mTOR pathway in PANC-1 and BxPC-3 cells ([Figure 4A](#)). In addition, we subsequently evaluated the effect of harmine plus gemcitabine on the AKT/mTOR pathway in pancreatic cancer cells. As shown in [Figure 4B](#), in PANC-1 and BxPC-3

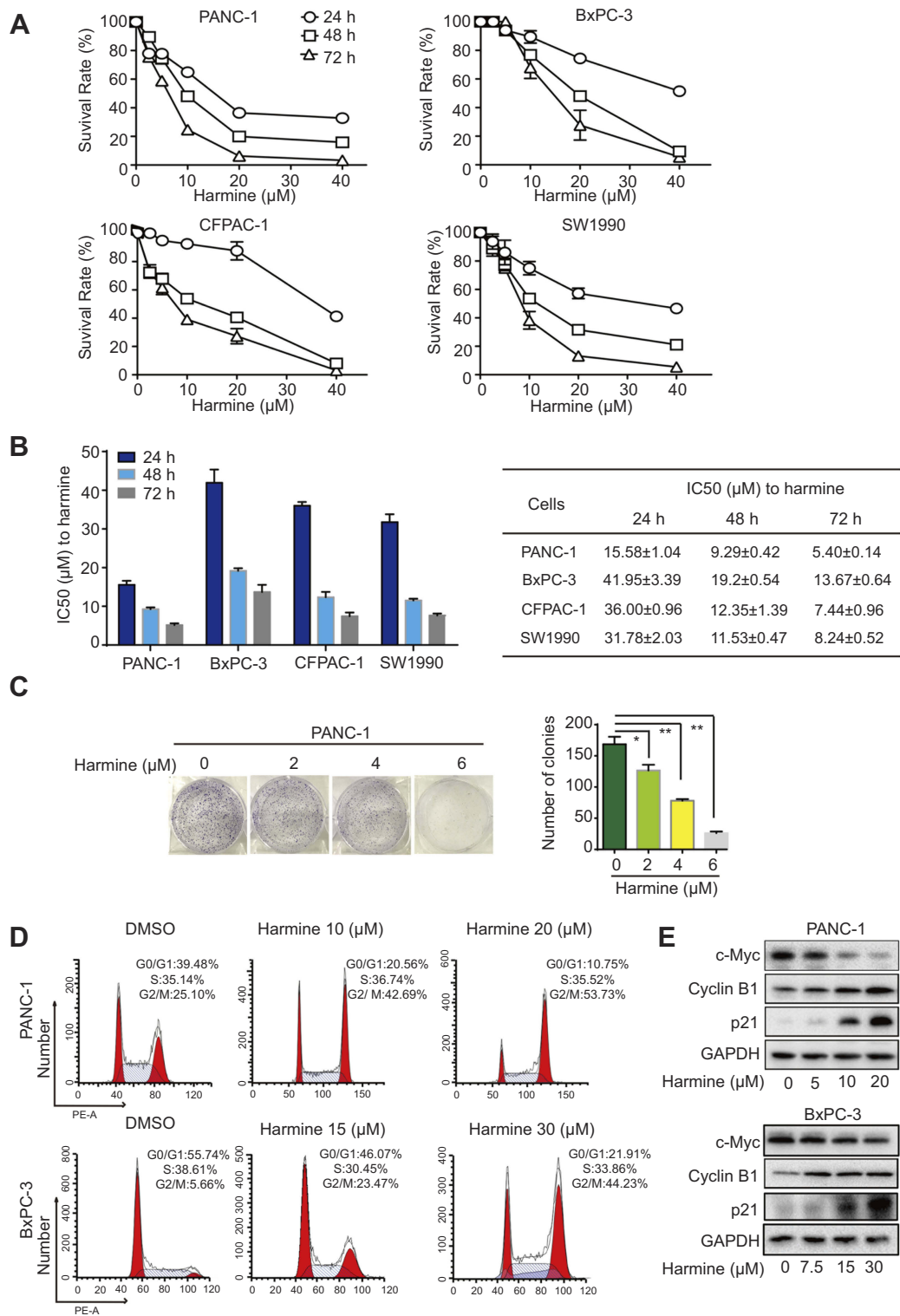
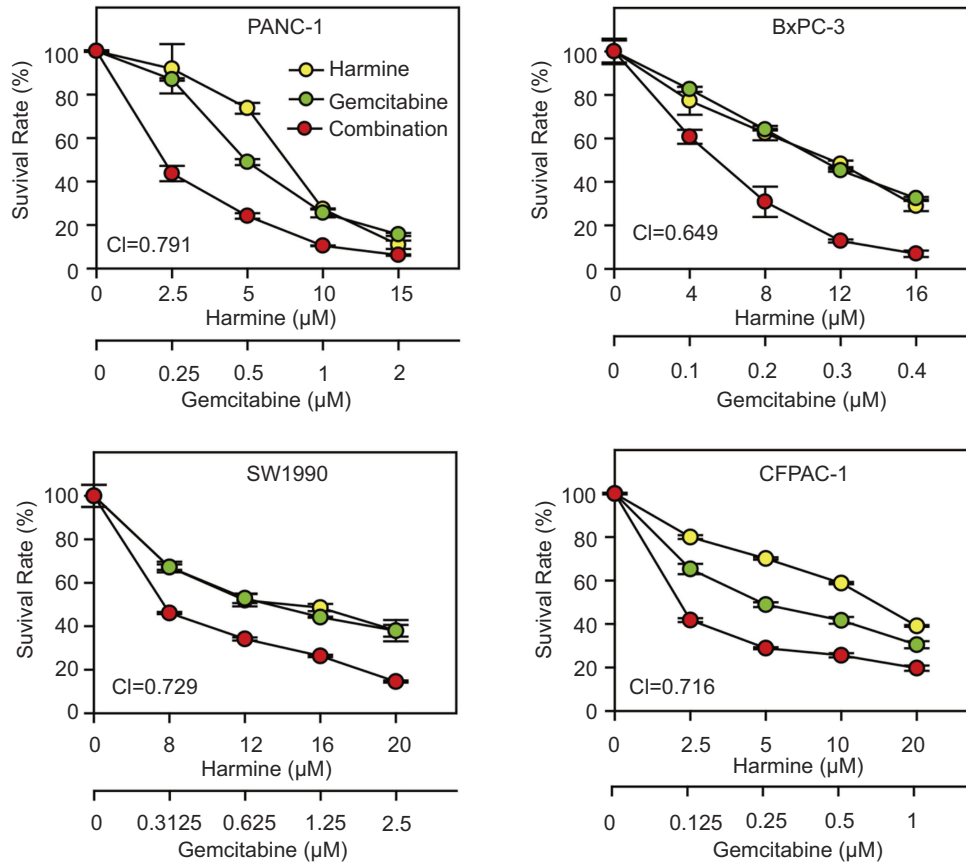


Figure 1 The anti-proliferation effect of harmine in pancreatic cancer cells. **(A)** Harmine significantly inhibited the proliferation of four pancreatic cancer cells, including PANC-1, CFPAC-1, SW-1990 and BxPC-3. Pancreatic cancer cells were treated with harmine at indicated concentrations for 24, 48 and 72 h, SRB assay was used to detect the proliferation of pancreatic cancer cells. **(B)** The IC50 of harmine in four pancreatic cancer cells were calculated and presented as means ± SD. **(C)** PANC-1 cells were treated with harmine at the indicated concentrations and cell clone was counted. **p*<0.05, ***p*<0.01. **(D)** PANC-1 and BxPC-3 cells were incubated with harmine at the indicated concentrations for 24 h, and cell cycle was detected by PI staining. **(E)** PANC-1 and BxPC-3 cells were incubated with harmine at the indicated concentrations for 24 h, and the expression of c-myc, cyclin B1 and p21 were detected by Western blotting.

Abbreviations: IC50, half maximal inhibitory concentration; PI, propidium iodide; SD, standard deviation.

A



B

PANC-1 cells			BxPC-3 cells		
Harmine (μM)	Gem (μM)	CI	Harmine (μM)	Gem (μM)	CI
2.5	0.25	0.794	4	0.1	0.691
5	0.5	0.631	8	0.2	0.655
10	1	0.786	12	0.3	0.672
15	2	0.952	16	0.4	0.577

SW1990 cells			CFPAC-1 cells		
Harmine (μM)	Gem (μM)	CI	Harmine (μM)	Gem (μM)	CI
8	0.3125	0.722	2.5	0.125	0.545
12	0.625	0.760	5	0.25	0.403
16	1.25	0.819	10	0.5	0.716
20	2.5	0.616	20	1	1.201

Figure 2 Harmine plus gemcitabine synergistically suppressed the proliferation of pancreatic cancer cells. **(A)** Pancreatic cancer cells were incubated with harmine and/or gemcitabine at indicated concentrations for 72 h, and the cytotoxicity of harmine plus gemcitabine was measured by SRB assay. **(B)** The CI values were calculated using Calcsyn.

Abbreviations: CI, combination index; SRB, Sulforhodamine B.

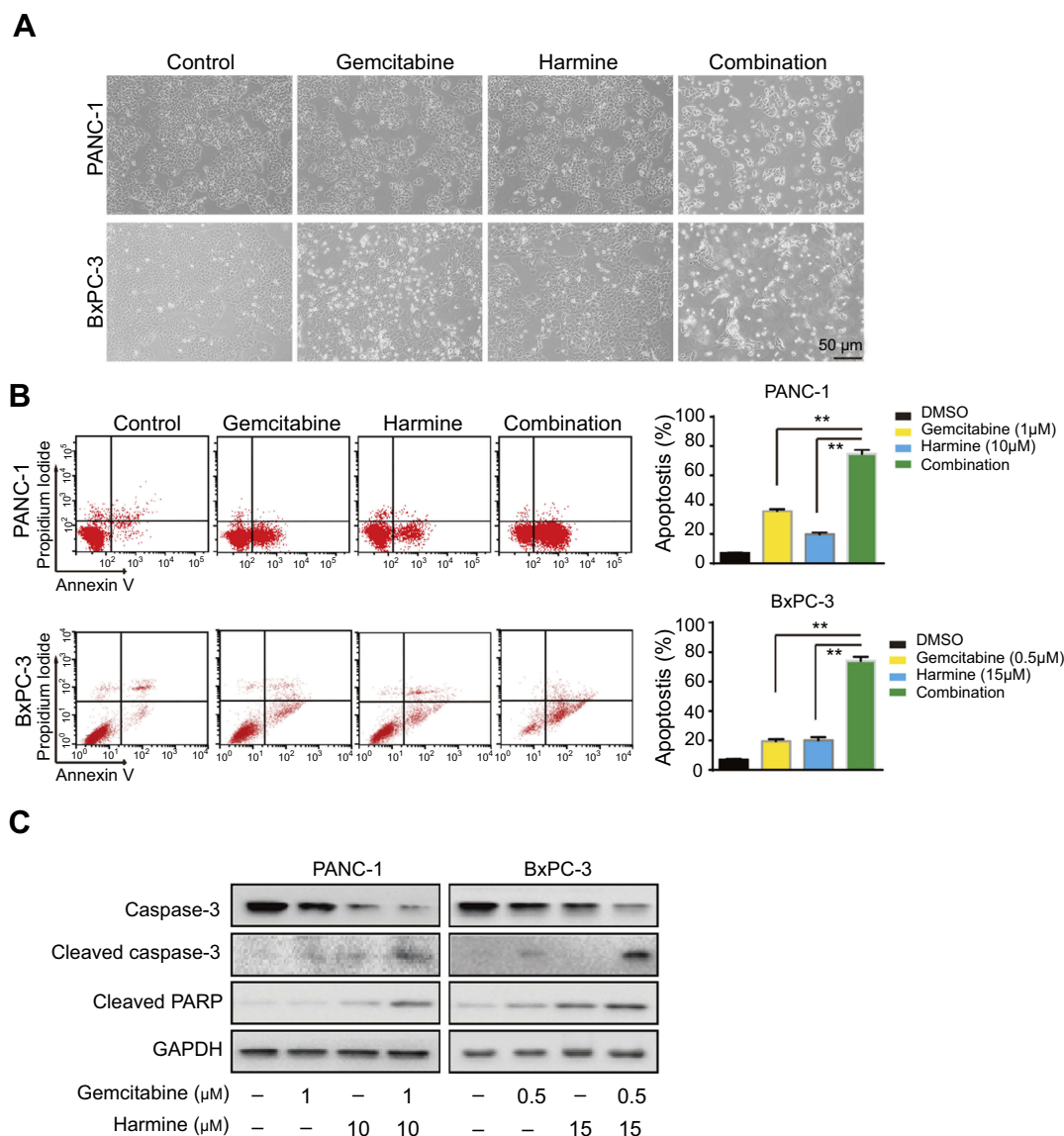


Figure 3 Harmine plus gemcitabine induced enhanced apoptosis in pancreatic cancer cells. **(A)** PANC-1 cells were treated with harmine (10 μM) and/or gemcitabine (1 μM), and BxPC-3 cells were treated with harmine (15 μM) and/or gemcitabine (0.5 μM) for 48 h, and cell morphological changes were observed using a scanning electron microscope; scale bar =50 μm. **(B)** Pancreatic cancer cells were incubated with harmine and/or gemcitabine at indicated concentrations for 48 h, and cells were collected and detected by Annexin V - FITC apoptosis detection kit. PANC-1 cells were detected by BD FACSCanto II, and BxPC-3 cells were detected by BD FACSCalibur Flow cytometer. **(C)** Pancreatic cancer cells were incubated with harmine and/or gemcitabine at the indicated concentrations for 48 h, and cells were collected and the expression of apoptosis related proteins were detected by Western blotting.

Abbreviation: DMSO, dimethyl sulphoxide.

cells, treatment with harmine plus gemcitabine significantly inhibited the expression of the members of the AKT/mTOR signaling pathway, including p-AKT (ser473), p-mTOR (Ser2448) and p-P70S6K.

Discussion

Pancreatic cancer is associated with a very poor prognosis, highlighted by the close parallel between the incidence and mortality associated with this disease. Gemcitabine has been a standard of chemotherapy for the treatment of metastatic pancreatic cancer.¹⁹ An array of different agents (eg, anti-

metabolites, nucleoside analogs, and DNA intercalating compounds), have been used against pancreatic cancer, alone or in combination, with limited improvement observed in patient survival.²⁰ Thus, the evolution of chemotherapy for pancreatic cancer is urgently warranted. In our study, we evaluated the anti-proliferative activity of harmine in four pancreatic cancer cell lines, including PANC-1, CFPAC-1, SW1990 and BxPC-3. Our data indicated that harmine inhibited the proliferation of pancreatic cancer cells, with the IC₅₀ ranging between 5.40 μM and 13.67 μM. Furthermore, harmine enhanced the anti-proliferative and apoptotic induction activity of

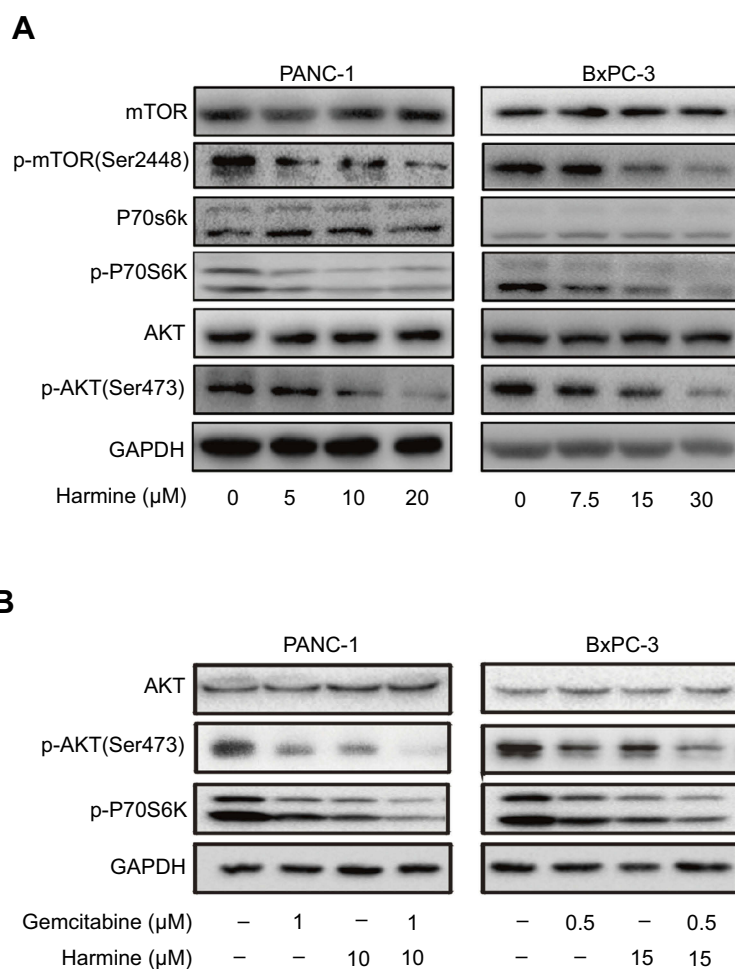


Figure 4 Harmine plus gemcitabine suppressed AKT/mTOR pathway. **(A)** Pancreatic cancer cells were treated with harmine at the indicated concentrations for 24 h, and cells were collected and the expression of AKT/mTOR pathway proteins were detected by Western blotting. **(B)** Pancreatic cancer cells were incubated with harmine and/or gemcitabine at the indicated concentrations for 24 h, and cells were collected and the expression of AKT/mTOR pathway proteins were detected by Western blotting. **Abbreviations:** P70S6K, P70Ribosomal protein S6 kinase; mTOR, mammalian target of rapamycin.

gemcitabine in pancreatic cancer cells. Our findings provide a novel strategy for the use of harmine in the treatment of pancreatic cancer. Moreover, the combination of harmine plus gemcitabine may be an effective combination regimen against this disease.

Patients with advanced pancreatic cancer are linked to a poor prognosis. Furthermore, there has been no improvement in patient survival since the introduction of gemcitabine in 1996.²¹ Pancreatic cancer remains one of the most deadliest types of cancer, with extremely low survival rates. This is attributed to delayed diagnosis and the development of resistance to standard therapies.²² The AKT/mTOR pathway is involved in mechanisms of resistance to gemcitabine in pancreatic cancer cells.²³ Furthermore, the development of resistance to gemcitabine in breast cancer cells is mainly mediated by activation of the AKT/mTOR signaling pathway.²⁴ In the present study, we showed that harmine suppressed the AKT/mTOR pathway, and harmine plus

gemcitabine also significantly suppressed the AKT/mTOR signaling pathway. Thus, harmine may enhance the anti-cancer activity of gemcitabine via suppressing the AKT/mTOR pathway. Therefore, our data indicated that the combination of gemcitabine with harmine may be an effective therapeutic regimens for the treatment of patients with gemcitabine-resistant pancreatic tumors. Although gemcitabine exhibits a good toxicity profile, myelosuppression remains the most common adverse effect associated with this agent.²⁵ Recently, it was reported that harmine may enhance the trabecular bone mass and osteogenic responses, and increased the number of preosteoclasts.²⁶ Hence, we suggest that harmine plus gemcitabine may be an attractive option for sensitization of cells to anti-pancreatic cancer activity. Moreover, this regimen may reverse the myelosuppression induced by gemcitabine during the treatment of pancreatic cancer. However, further in-vivo studies are warranted to test the hypothesis.

Conclusion

In conclusion, harmine may be a promising drug for the treatment of pancreatic cancer. The combination of harmine with gemcitabine may be an effective therapeutic strategy, offering synergistic efficacy and low toxicity in patients with pancreatic cancer. Furthermore, gemcitabine plus harmine may be an efficacious therapeutic regimen for patients with gemcitabine-resistant pancreatic tumors.

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Disclosure

The authors report no conflicts of interest in this work.

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