

Akt inhibitor MK-2206 reduces pancreatic cancer cell viability and increases the efficacy of gemcitabine

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Abstract. The PI3K/Akt pathway is an attractive therapeutic target in the treatment of pancreatic cancer, as it was demonstrated to be aberrantly regulated in pancreatic cancer cells. The present study aimed to investigate the therapeutic potential of the novel Akt inhibitor MK-2206 in human pancreatic cancer cell lines. Pancreatic cancer cell survival following MK-2206 treatment was assessed using the Cell Counting Kit-8 (CCK-8) assay, colony formation and determination of the apoptotic rate by flow cytometry following annexin-V-fluorescein isothiocyanate/propidium iodide staining. The effects of MK-2206 alone or in combination with gemcitabine on pancreatic cell proliferation were assessed using the CCK-8 assay. Western blotting was used to examine the effects of the two drugs on Akt protein expression. The results demonstrated that MK-2206 inhibited the proliferation and induced apoptosis of the Mia PaCa-2 and Panc-1 pancreatic cancer cell lines. In addition, CCK-8 cytotoxicity test showed that combined administration of MK-2206 with gemcitabine enhanced the cytotoxic efficacy of gemcitabine. Furthermore, a low dose of MK-2206 (1 μ M) combined with gemcitabine was enough to inhibit Akt phosphorylation. Taken together, these results provided some insight into the underlying mechanism of the anticancer effects of MK-2206 on pancreatic cancer cells.

Introduction

Pancreatic cancer is one of the most lethal human malignancies worldwide (1). To date, the causes underlying pancreatic cancer are still unknown, although certain risk factors have been identified. The possible risk factors for pancreatic cancer include sex (male), age (≥ 50), smoking, alcohol abuse, obesity, physical activities, diabetes, chronic

pancreatitis and genetic alterations (2). Patients diagnosed with pancreatic cancer typically have a poor prognosis (1,2). Although mortality from the most common types of cancer has declined in the past few decades, the mortality of patients with pancreatic cancer remains high with a 5-year survival rate of only 6% (1). Surgical resection is the only potential curative therapy for pancreatic cancer. In the majority of cases, the disease has already metastasized to distant organs at the time of diagnosis (1,2). Gemcitabine (dFdC) is a deoxycytidine nucleoside analog, whose anti-proliferative properties are dependent on several inhibitory actions on DNA synthesis, blocking cell cycle progression at the G₁/S-phase boundary (3). Gemcitabine has been recognized as the standard first-line chemotherapy drug for pancreatic cancer. However, a previous study showed that gemcitabine can increase patient median survival by only 6 months (4). It is therefore crucial to develop novel therapeutic options and effective treatments for this disease.

It has been demonstrated that the PI3K/Akt pathway is excessively activated in various types of cancer, including pancreatic cancer (5). Furthermore, up to 60% of pancreatic cancer tissues and most pancreatic cancer cell lines exhibit increased Akt activity (6). Previous studies have reported that Akt is a major mediator of cell survival and apoptosis through the regulation of pro-survival and antiapoptotic proteins, including Bcl-XL and NF- κ B in both normal and neoplastic cells (7,8). Modulation of the Akt signaling pathway may therefore be considered a promising therapeutic approach for the treatment of pancreatic cancer.

MK-2206 is an allosteric Akt inhibitor that has been approved as an anti-cancer agent and that is administered orally in patients with cancer (such as renal cell carcinoma, parotid adenocarcinoma and colorectal cancer) (9). Previous studies have demonstrated that MK-2206 can suppress tumor growth (such as renal cell carcinoma, parotid adenocarcinoma and colorectal cancer) and enhance the antitumor efficacy of conventional chemotherapeutic agents, including docetaxel and carboplatin (10,11). However, to the best of our knowledge, only a few studies (10,11) have investigated the effect of MK-2206 on pancreatic cancer. The present study aimed to evaluate the antitumor effect of MK-2206 in pancreatic cancer cell lines in order to determine whether it may be considered as a promising therapeutic agent for the treatment of patients with pancreatic cancer.

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Materials and methods

Cell culture. The human pancreatic cancer lines Mia PaCa-2, Panc-1, BxPC-3, AsPC-1, Capan-2, CFPAC-1 and SW1990 were obtained from the American Type Culture Collection. All cell lines (Mia PaCa-2, Panc-1, Capan-2, CFPAC-1 and SW1990) were routinely maintained in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences) or RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) (BxPC-3 and AsPC-1) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere of 5% CO₂ at 37°C.

Reagents and antibodies. MK-2206 and gemcitabine were purchased from Selleck Chemicals. MK-2206 was dissolved in DMSO according to the instruction. Gemcitabine was dissolved in water according to the instruction. The primary antibodies against phosphorylated (p)-Akt (ser473) (cat. no. 3787), total (T)-Akt (cat. no. 4691) and β -actin (cat. no. 12262) were purchased from Cell Signaling Technology, Inc. Anti-rabbit IgG, HRP-linked secondary antibody (cat. no. 7074) were purchased from Cell Signaling Technology, Inc.

Western blotting. Pancreatic cells were lysed with 2X SDS lysis buffer. Bicinchoninic acid (EpiZyme) was used to quantify proteins. The amount of proteins loaded per lane was 20 μ g. Proteins were separated by SDS-PAGE (separating gel concentration, 10%; stacking gel concentration, 10%) and transferred onto nitrocellulose membranes (Axygen; Corning, Inc.). Membranes were blocked with 5% milk in Tris-buffered saline (TBS) for 1 h at room temperature, and incubated overnight with the primary antibodies (p-Akt (1:1,000), T-Akt (1:1,000) and β -actin (1:1,000) at 4°C. After incubation with HRP-conjugated secondary antibodies (anti-rabbit secondary antibody for p-Akt and T-Akt, 1:5,000) for 1 h at room temperature, the membranes were washed 3 times with TBS for 10 min. Bands were detected using enhanced chemiluminescence detection kit (EMD Millipore).

Cell viability assay. Mia PaCa-2 and Panc-1 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the medium was replaced by various concentrations of drugs, and the cells were incubated for another 48 h. Cell viability was determined using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). The amount of CCK-8 reagent added per well was 10 μ l. Cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Absorbance was measured at 450 nm using a microplate reader.

Colony-formation assay. To assess the effect of MK-2206 on the colony formation of Mia PaCa-2 and Panc-1 cell lines, cells were seeded in 3.5 cm dishes at a density of 1×10^3 cells/dish. The medium was renewed every three days until visible colonies were formed for one week. Cells were fixed with anhydrous methanol for 15 min and stained with 1% crystal violet for 10 min at room temperature. Colonies were captured with a camera.

Apoptosis detection. The apoptotic rates of Mia PaCa-2 and Panc-1 cell lines following treatment with MK-2206 were assessed by flow cytometry with Annexin-V/propidium iodide (PI) staining according to the manufacturer's instructions (cat. no. FA101-01; TransGen Biotech). Apoptotic rates were subsequently determined using a flow cytometer (LSRFortessa X-20; BD Biosciences).

Statistical analysis. All data were presented as the means \pm standard deviation. Three independent repeats were conducted for each experiment. Comparison between the groups was calculated using a two-tailed Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MK-2206 inhibits Akt activation in pancreatic cancer cell lines. The expression of p-Akt was examined in human pancreatic cancer cell lines. The results demonstrated that different levels of Akt were activated in different pancreatic cancer cell lines (Fig. 1A). The effect of MK-2206 on Akt phosphorylation was subsequently examined. Treatment with MK-2206 was performed at 0.1 and 1 μ M for 48 h. As presented in Fig. 1B, MK-2206 treatment reduced the expression p-Akt in all pancreatic cancer cell lines. No changes in the levels of total Akt protein were observed. These results demonstrated that MK-2206 inhibited Akt phosphorylation in pancreatic cancer cells.

MK-2206 inhibits the proliferation and induces the apoptosis of pancreatic cancer cells. CCK-8 assay was performed to examine the effects of MK-2206 on Mia PaCa-2 and Panc-1 cell proliferation. As presented in Fig. 2A, MK-2206 inhibited cell proliferation of human cancer cell lines. Furthermore, colony formation assay was performed to assess the effects of MK-2206 on the viability of Mia PaCa-2 and Panc-1 cells. The results demonstrated that the number of colonies formed following treatment with MK-2206 was reduced in both cell lines (Fig. 2B).

To assess MK-2206-induced apoptosis, Mia PaCa-2 and Panc-1 cells were treated with DMSO (0.1%) or 1 μ M MK-2206 for 48 h, and the percentage of annexin V-PI positive cells was determined. The results demonstrated that treatment with MK-2206 for 48 h significantly induced apoptosis in the two pancreatic cancer cell lines (Fig. 2C and D).

MK-2206 increases the cytotoxic effects of gemcitabine in human pancreatic cancer cells. Gemcitabine has been extensively used in the treatment of pancreatic cancer (3). As presented in Fig. 3A and B, MK-2206 and gemcitabine co-treatment for 48 h significantly decreased cell proliferation compared with the gemcitabine and DMSO group. These data suggested that MK-2206 may enhance the cytotoxicity induced by gemcitabine in pancreatic cancer cells.

MK-2206 inhibits gemcitabine-induced Akt activation in pancreatic cancer cell lines. A previous study demonstrated that gemcitabine induces Akt activation in pancreatic cancer cells (12). Activation of the Akt survival pathway may therefore block the therapeutic efficacy of

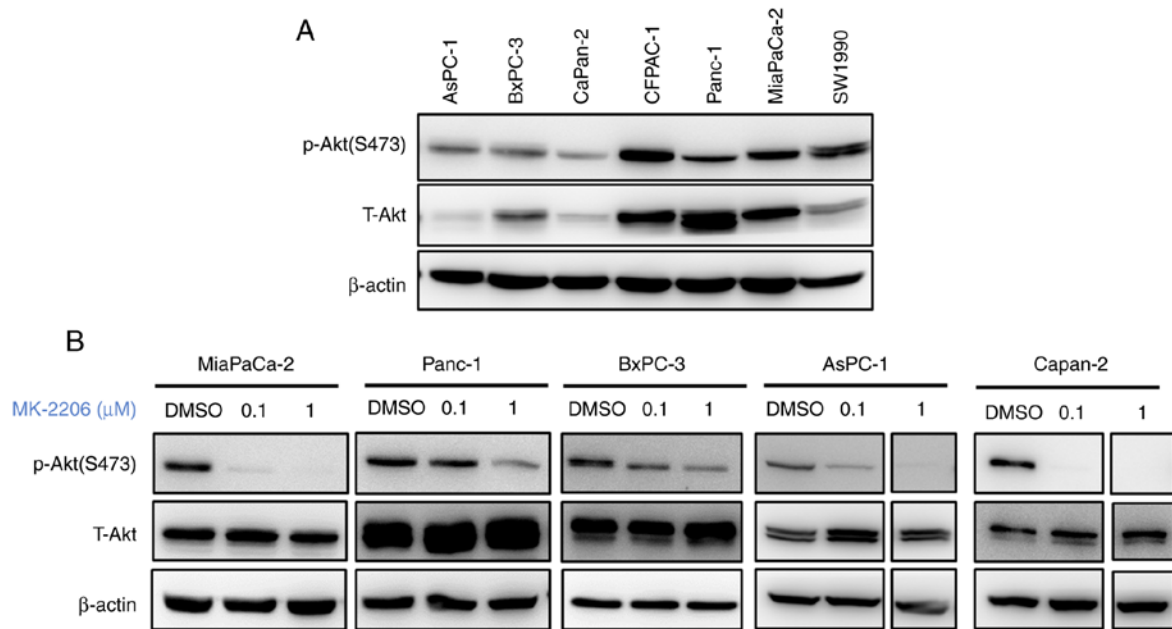


Figure 1. MK-2206 inhibits Akt phosphorylation in pancreatic cancer cell lines. (A) Expression of p-Akt and T-Akt was analyzed by western blotting in human pancreatic cancer cell lines. (B) Mia PaCa-2, Panc-1, BxPC-3, AsPC-1 and Capan-2 cells were treated with MK-2206 (0.1 and 1 μ M) for 48 h, and the expression of p-Akt and T-Akt was measured by western blotting. β -actin was used as a loading control. n=3. p, phosphorylated; T, total.

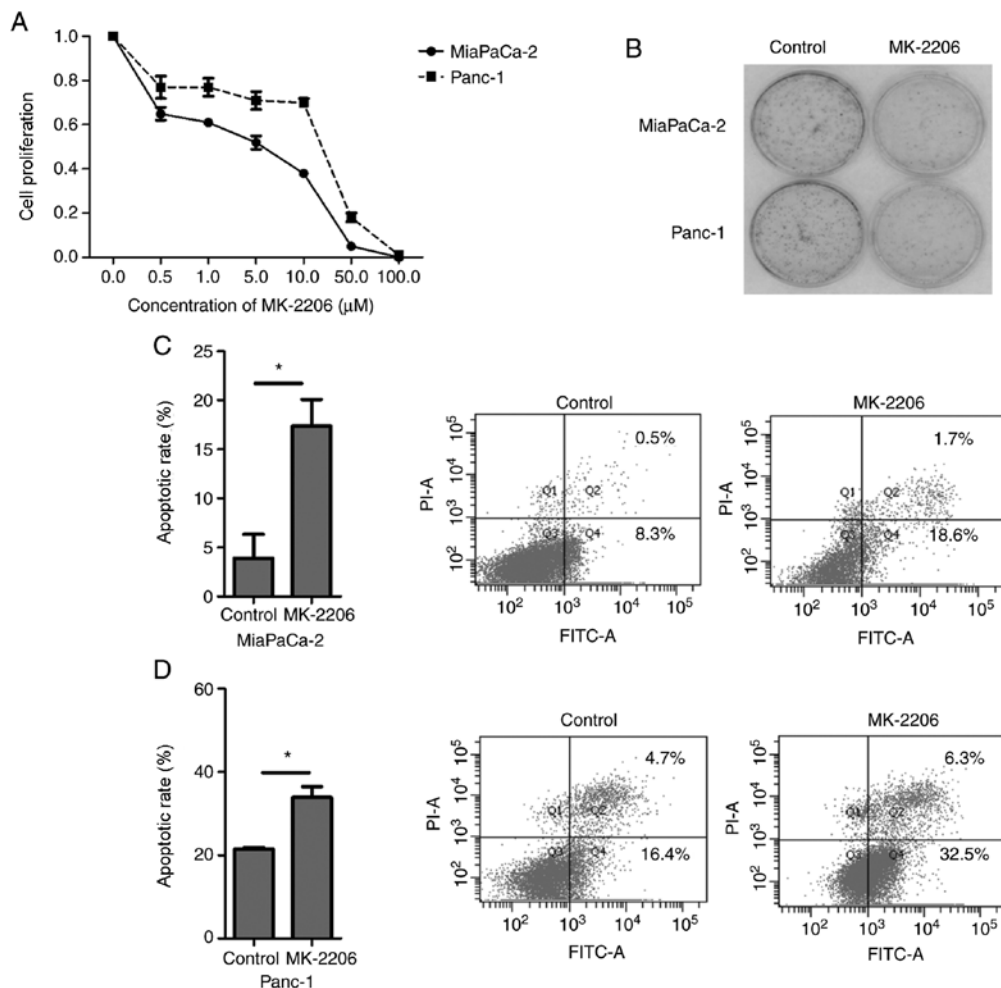


Figure 2. MK-2206 inhibits the proliferation and induces the apoptosis of pancreatic cancer cells. (A) Mia PaCa-2 and Panc-1 cells were treated with MK-2206 at the indicated concentration for 48 h. Cell proliferation was evaluated using the Cell Counting Ki-8 assay. (B) Colony formation was quantified 10 days post-treatment with MK-2206. (C) Mia PaCa-2 and (D) Panc-1 cells were treated with 1 μ M MK-2206 for 48 h prior to assessing the apoptotic rate by flow cytometry using annexin-V/PI staining. n=3. PI, propidium iodide.

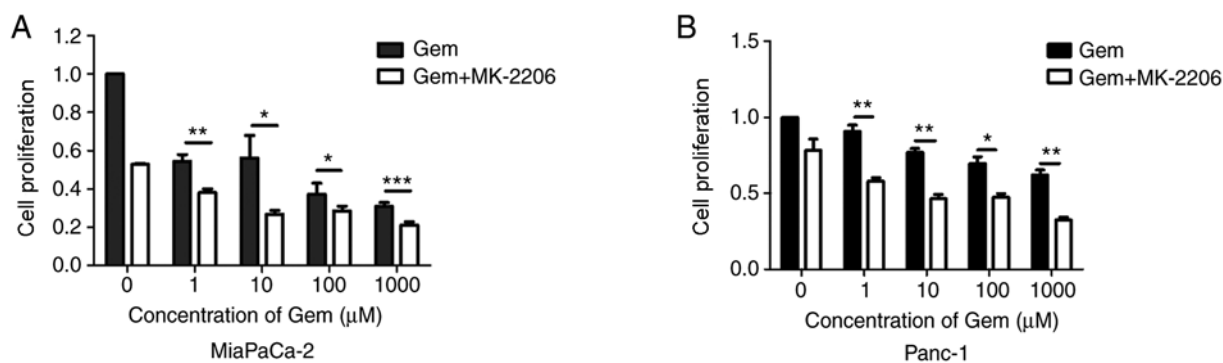


Figure 3. Co-treatment with MK-2206 and Gem increases the cytotoxic effect of gemcitabine. (A) Mia PaCa-2 and (B) Panc-1 cell lines were simultaneously treated with 1 μ M MK-2206 and Gem at the indicated concentration for 48 h. Cell proliferation was evaluated using the Cell Counting kit 8 assay. $n=3$. * $P<0.05$, ** $P<0.01$ and *** $P<0.005$ vs. gemcitabine treatment group. Gem, gemcitabine.

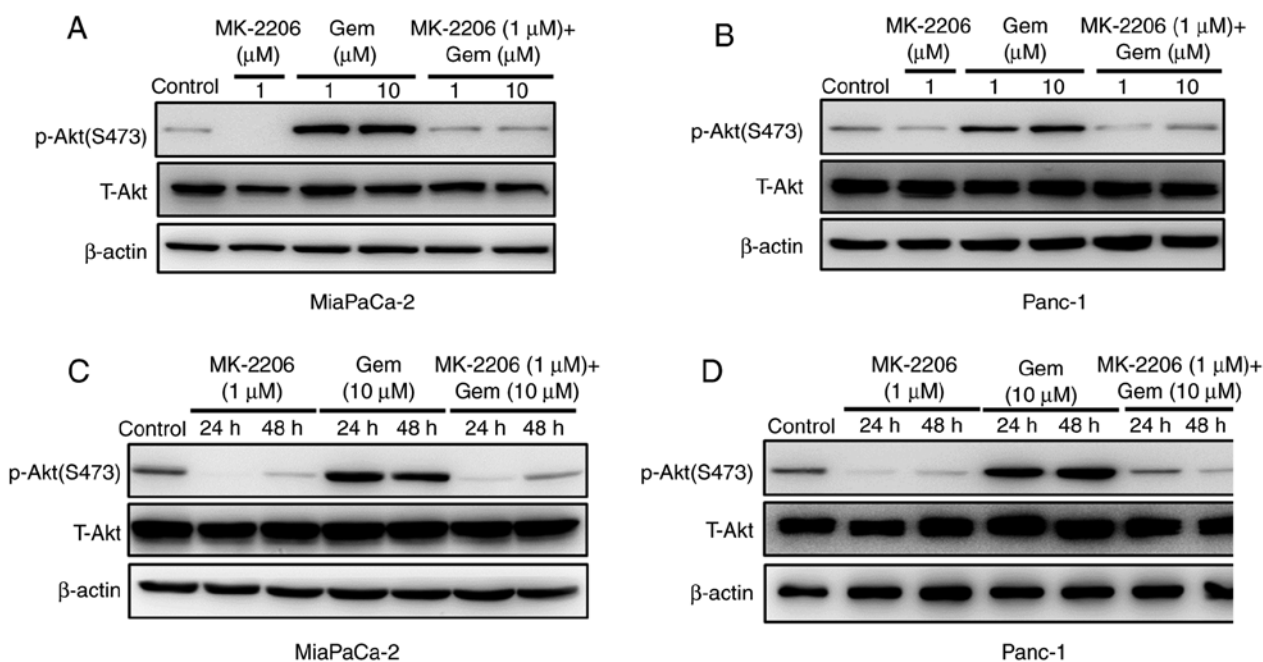


Figure 4. Co-treatment with MK-2206 and Gem inhibits the gemcitabine-induced Akt activation. (A and B) Cells were treated with Gem (1 or 10 μ M) in the presence of MK-2206 (1 μ M) for 48 h. MK-2206 inhibited Akt phosphorylation induced by Gem in (A) Mia PaCa-2 and (B) Panc-1 cell lines. (C and D) Time-dependent effects of MK-2206 and Gem on p-Akt and T-Akt expression in (C) Mia PaCa-2 and (D) Panc-1 cell lines. Cells were treated with 1 μ M MK-2206 alone, 10 μ M Gem alone or both agents for 24 and 48 h. The expression levels of p-Akt and T-Akt were determined by western blotting. β -actin was used as a loading control. $n=3$. Gem, gemcitabine; p, phosphorylated; T, total.

gemcitabine (10). To investigate whether MK-2206 may inhibit gemcitabine-induced Akt activation in pancreatic cancer cells, p-Akt expression in Mia PaCa-2 and Panc-1 cell lines was assessed following treatment with gemcitabine alone (1 or 10 μ M) or in combination with 1 μ M MK-2206 for 24 and 48 h. The results demonstrated that the expression of p-Akt (Ser473) was increased after treatment with 1 and 10 μ M gemcitabine for 48 h in Mia PaCa-2 and Panc-1 cell lines. However, gemcitabine-induced Akt phosphorylation was inhibited by 1 μ M MK-2206 in these two pancreatic cancer cell lines (Fig. 4A and B). In addition, 10 μ M gemcitabine treatment for 24 and 48 h increased Akt phosphorylation in Mia PaCa-2 and Panc-1 cell lines, and gemcitabine-induced Akt phosphorylation was inhibited by 1 μ M MK-2206 treatment for 24 and 48 h in these two pancreatic cancer cell lines (Fig. 4C and D).

Discussion

MK-2206 is a highly selective allosteric inhibitor of Akt that binds to the pleckstrin-homology domain of Akt, inducing a conformational change that prevents Akt localization to the plasma membrane and its subsequent activation (13). In the present study, human pancreatic cancer cell lines Mia PaCa-2, Panc-1, CFPAC-1 and SW1990 exhibited higher p-Akt expression compared with BxPC-3, AsPC-1 and Capan-2 cell lines. In addition, a previous study from our laboratory demonstrated that Mia PaCa-2 and Panc-1 cell lines were more resistant to gemcitabine compared with CFPAC-1 and SW1990 cell lines (data not shown). Thus, Mia PaCa-2 and Panc-1 cell lines were selected for subsequent experiments in the present study. The results of the present study demonstrated that MK-2206 decreased p-Akt expres-

sion and reduced cell proliferation and colony formation in Mia PaCa-2 and Panc-1 cell lines. In addition, MK-2206 stimulated apoptosis in Mia PaCa-2 and Panc-1 cells. These results were consistent with previous studies on other types of cancer, including gastric cancer, neuroblastoma and thyroid cancer (14-16).

It is worth noting that the inhibition rate of MK-2206 in pancreatic cancer cells in Fig. 3A and B disagree with those of Fig. 2. This may have occurred due to the long-term use of the cell lines, as cellular viability decreases and cell senescence level increases with the extension of the passage number.

Gemcitabine, used alone or in combination with other therapeutic agents, is the first-line chemotherapy strategy for the treatment of locally advanced or metastatic pancreatic cancer (4,17). However, most patients do not respond well and develop chemoresistance to gemcitabine (18). It has been reported that gemcitabine can activate the Akt signaling pathway in pancreatic cancer cells via the overproduction of reactive oxygen species, which reduces the anti-tumor responses and inhibits the therapeutic efficacy of gemcitabine (12). In the present study, MK-2206 treatment in combination with gemcitabine abolished the gemcitabine-induced Akt activation and increased the cytotoxic effect of gemcitabine. These findings were consistent with a previous study reporting that MK-2206 can sensitize human cancer cells to numerous chemotherapeutic agents, including doxorubicin and fluorouracil (10). Previous clinical phase 1 trial showed that MK-2206 combination with carboplatin and paclitaxel, docetaxel, or erlotinib, was well-tolerated at doses that inhibit Akt signaling (19). Furthermore, a number of ongoing or completed phase 2 trials have demonstrated that numerous tumor types are responsive to MK-2206 alone or in combination with other drugs (20,21). Combining gemcitabine with MK-2206 may therefore be considered as a promising therapeutic strategy for the treatment of patients with pancreatic cancer.

In conclusion, the results of the present study demonstrated that MK-2206 inhibited Akt phosphorylation and attenuated pancreatic cancer cell proliferation. In addition, the combination of MK-2206 with gemcitabine enhanced pancreatic cancer cell proliferation inhibition, which may have been due to the MK-2206-mediated inhibition of gemcitabine-induced Akt activation. It is worth noting that the toxicity of MK-2206 on normal human pancreatic ductal epithelium cells was not examined in the present study. A previous study reported that MK-2206 was well tolerated in patients and that its toxic and side effects were acceptable (9). The combination of MK-2206 with gemcitabine may therefore help overcome chemoresistance in pancreatic cancer. Further *in vivo* investigation using patient-derived xenograft models is required to confirm these findings.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZW and GL conceived and designed the experiments. GL interpreted the data, performed the statistical analysis and analyzed the results. ZW and GL wrote the manuscript. ZW and ZQ revised the manuscript. ZQ made substantial contributions to conception and design of the study. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy of integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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