Original Article

Nobiletin Inhibits Cell Growth, Migration and Invasion, and Enhances the Anti-Cancer Effect of Gemcitabine on Pancreatic Cancer Cells

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Abstract

Natural products are very promising adjuvants with a variety of biological activities. Nobiletin, a citrus polymethoxyflavone, has been shown to exert an anticancer effect in various cell lines. In this study, we investigated the effects of nobiletin on cell viability, sphere formation, migration and invasion of pancreatic cancer cells, and the underlying mechanisms. Our results demonstrate that nobiletin significantly inhibited PANC-1 cell migration and invasion, and these effects were associated with downregulation of MMP-2. We also found that nobiletin, in a low concentration, exhibited a strong inhibitory effect on sphere formation. The potential molecular mechanisms were related to significant downregulation of p-mTOR and p-STAT3. Furthermore, we found that nobiletin combined with gemcitabine synergistically inhibited PANC-1 cell viability and sphere formation. The underlying mechanisms of the synergistic inhibition on growth were associated with decreases in p-STAT3 expression. Overall, our results suggest that nobiletin may be a promising candidate for pancreatic cancer adjuvant treatment.

Keywords

nobiletin, gemcitabine, STAT3, mTOR, pancreatic cancer

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Pancreatic cancer is one of the most aggressive and lethal cancers worldwide. It was estimated that this disease caused more than 45 000 deaths in the United States. In contrast to other cancer types, pancreatic cancer has the lowest 5-year relative survival rate of only 9%.[1-3] Gemcitabine, a nucleoside analog, is the first-line FDA-approved chemotherapy drug for treating pancreatic cancer.[4,5] However, gemcitabine has a low response rate with limited progression-free survival in patients.[6] Chemoresistance and toxic side effects also limit the clinical success of gemcitabin.[6] Hence, it is urgent to develop novel preventive and/or therapeutic approaches for better control and management of this aggressive malignant tumor disease.

Compounds derived from natural sources have been shown to inhibit signaling pathways important for cancer cell growth and survival.[7-9] Polymethoxyflavones (PMFs) are mainly derived from the *Citrus* species (family Rutaceae), especially from *C. sinensis* and *C. reticulata*.[10,11] Some studies have demonstrated a wide range of biological activities of PMFs, such as anti-inflammatory,[12] anti-cancer,[13,14] and preventing obesity[15] effects. Nobiletin (Figure 1(A)), one of the widely known citrus polymethoxyflavones, has anti-proliferative, anti-angiogenesis, and apoptotic effects in various cancer types, including pancreatic cancer.[14,16-19] Moreover, nobiletin exhibits a great ability for enhancing the efficacy of anticancer agents. One group of researchers demonstrated that nobiletin enhances the efficacy of paclitaxel in ABCB1 overexpressing cancer cells by inhibiting the AKT/ERK/Nrf2 pathway,[20] and studies conducted by another group of researchers indicated

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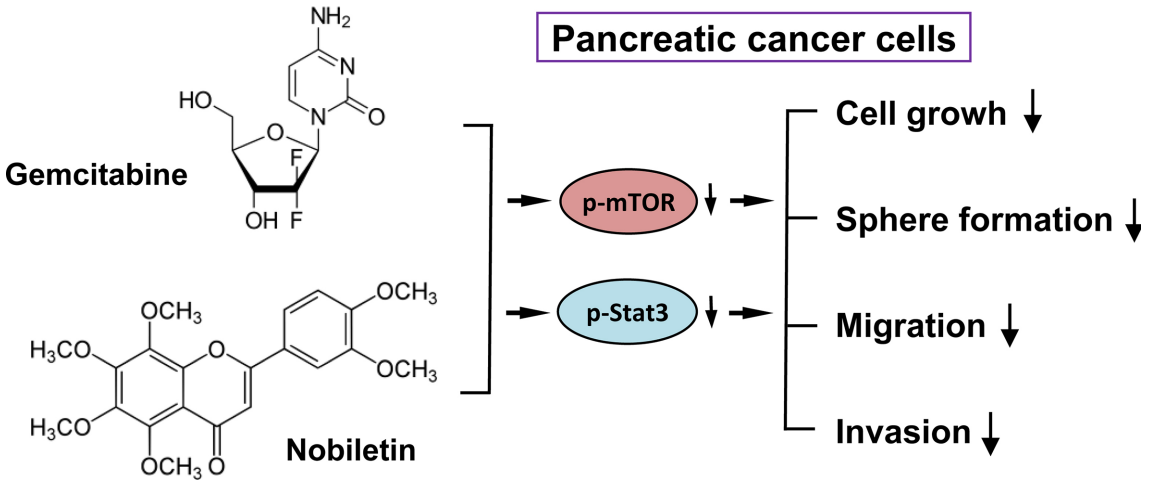

that nobiletin, in combination with atorvastatin, acts synergistically to inhibit colon carcinogenesis.[21] However, the effect of nobiletin in combination with gemcitabine on pancreatic cancer cells has not been studied.

In the present study, we aimed to determine the potential effect of nobiletin on pancreatic cancer in vitro and reveal the possible molecular mechanisms. Our data demonstrated that nobiletin effectively inhibited pancreatic cancer cell survival, sphere formation, migration and invasion. The effects of nobiletin on pancreatic cancer cells were associated with decreases in phosphorylated mTOR (p-mTOR) and p-STAT3 phosphorylation. Moreover, the results of our experiments indicate that nobiletin enhances the effect of gemcitabine on pancreatic cancer cells. The results of our present study suggest that nobiletin may be a promising candidate for future development of pancreatic cancer adjuvant treatment.

Methods

Cell Culture and Reagents

PANC-1 and MIA CaPa-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Nobiletin was acquired from Dalian Meilun Biotechnology Co, Ltd (MB6570-S), and gemcitabine from LC Laboratories, USA. DMEM culture medium, penicillin/streptomycin, L-glutamine, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). PANC-1 cells were maintained in a DMEM culture medium containing 10% FBS that was supplemented with penicillin (100 units/ml)-streptomycin (100 μg/mL) and L-glutamine (300 μg/mL). Cultured cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and were passaged twice a week.

Cell Viability Assay

PANC-1 and MIA PaCa-2 cells were seeded on 96-well plates at a density of 5×10^3 per well and incubated at 37 °C for 24 hours. The cells were then treated with various concentrations of nobiletin (0, 120 μM) for 72 hours. After treatment, 100 μL of MTT solution (0.5 mg/mL in DMEM medium) was added to each of the wells and incubated at 37 °C for 4 hours. After careful remove of the medium, 100 μL DMSO was added to each well. The absorbance was recorded on a microplate reader at 490 nm.

Wound-Healing Assays

Cell migration ability was assessed using a wound-healing assay. In brief, cells were seeded at 1×10^6 cells/well in 6-well plates. After 24 hours, when the cells were 95% to 100% confluent, media was removed and the cell surface was scratched using a sterile 200 μL pipette tip. Floating cells were removed by washing twice with PBS. Cultivation was subsequently continued for 24 hours and 48 hours in 1% FBS medium. The cells were treated with different concentrations of nobiletin.

Transwell Analysis

Transwell invasion assay was performed in a 24-well transwell unit (8 μm pore size) which was coated with Matrigel matrix (Corning, USA) as described by the manufacturer. Cells were seeded at 4×10^5 cells/well in serum-free DMEM media (200 μL) in the top chamber, and treated with different concentrations of nobiletin for 24 hours. The lower chamber was filled with 800 μL DMEM supplemented with 20% FBS. The ability of migration was assessed in chambers without matrix. After incubation at 37 °C for 24 hours, cells were fixed with methanol and stained with 0.1% crystal violet. The number of cells was determined by counting randomly on each membrane.

Western Blot

PANC-1 cells were seeded at a density of 1×10^6 cells in 100 mm culture dishes and treated with nobiletin for 24 hours. After treatment, the cell lysates were prepared as described earlier. Proteins were subjected to sodium dodecyl sulfate

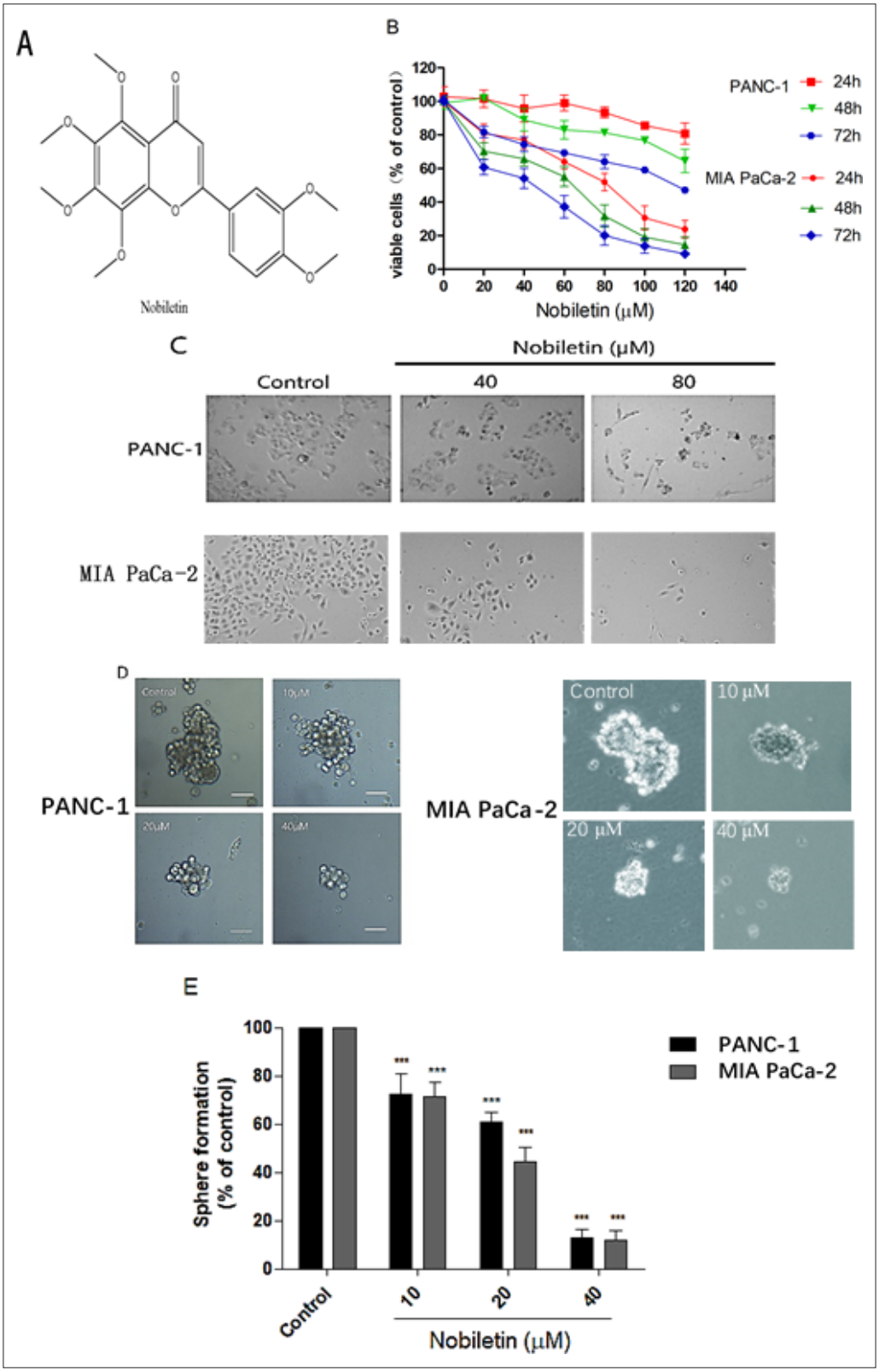

Figure 1. Effect of nobiletin on cell viability in PANC-1 and MIA PaCa-2 cells. (A) Chemical structures of nobiletin. (B) PANC-1 and MIA PaCa-2 cells were treated with nobiletin for 24 hours, 48 hours, and 72 hours, and cell viability was measured using MTT assay. (C) Cell morphology under a microscope treated with nobiletin for 72 hours. Effect of nobiletin on sphere formation in PANC-1 cells and MIA PaCa-2 cells. (D-E) Tumor sphere size and sphere formation rate were shown after treated with nobiletin for 14 days. Data shown are mean ± SEM, $n = 3$, $^{***}P < 0.001$ compared with the control group.

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After blocking nonspecific binding sites with blocking buffer, the membrane was incubated overnight at 4 °C with primary antibodies. β-Actin was used as a loading control. Following removal of the primary antibody, the membrane was washed 3 times with TBS (PBS containing 0.05% tween 20) buffer at room temperature and then incubated with fluorochrome conjugated secondary antibody. The membrane was then washed with TBS, 3 times. The final detection was carried out using Pierce™ ECL Western Blotting Substrate (Thermo, USA).

Sphere Formation Assay

For sphere formation assay, PANC-1 and MIA PaCa-2 cells were dissociated and passed through a 70 μm filter (BD, Falcon) to produce single cell suspensions. Then, 3000 cells/well were seeded and grown in a 24-well plate with Keratinocyte serum-free medium, supplemented with 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (BPE) (both from Gibco, USA) for 14 days.[22] The cells were treated with different concentrations of nobiletin. After that, the number of spheres was counted under a light microscope (Nikon Optiphot, Tokyo, Japan).

Transfection With STAT3 and mTOR siRNA

STAT3 siRNA, mTOR siRNA and scrambled negative control siRNA were from Cell Signaling Tech. PANC-1 cells were seeded in 6-well plates and incubated for 24 hours to allow adherence of cells. SiRNA was transfected into the cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instruction. The final concentration of siRNA was 100 nM. After 48 hours, the cells were harvested for expression assay or treating with nobiletin and gemcitabine.

Statistical Analysis

The interaction between nobiletin and gemcitabine was determined using a combination index (CI), which was calculated according to the median-effect principle, based on previous reports.[23,24] The equation for the isobologram was shown as CI = $A_1/A_2 + B_1/B_2$. A_1 and B_1 represent the doses of nobiletin and gemcitabine necessary to produce the same effect in combination, and A_2 and B_2 represent the individual doses of nobiletin and gemcitabine required to inhibit a given level of cell viability, respectively. If CI is <1, then the drugs are considered to act synergistically. If CI is >1 or =1, the drugs act in an antagonistic or additive manner, respectively. Statistical analyses were done by using the software InStat (GraphPad Software, Inc., La Jolla, CA, USA). All the data are presented as mean ± SEM. Experimental data analysis was carried out using a one-way ANOVA. A P value < 0.05 was considered statistically significant.

Results

Effects of Nobiletin on Cell Viability and Sphere Formation in Pancreatic Cancer Cells

In initial studies, the effect of nobiletin on cell viability and sphere formation in pancreatic cancer cells were determined. The structure of nobiletin is shown in Figure 1(A). PANC-1 and MIA PaCa-2 cells were treated with nobiletin at different concentrations (10, 120 μM) for 24 hours, 48 hours, and 72 hours, and then analyzed by MTT assay. As shown in Figure 1(B), treatment with nobiletin resulted in decreases in the viability of the cells in a dose-dependent and time-dependent manner. These results agree with previous studies.[19] With increase in time and concentration, the inhibitory effect of nobiletin on the 2 cell lines increased. Under the same conditions, nobiletin had a stronger inhibitory effect on MIA PaCa-2 cells than on PANC-1 cells. After treatment with nobiletin for 72 hours, the cell morphology observed by microscopy is shown in Figure 1(C). In addition, we determined the inhibitory effect of nobiletin on the formation of spheres in PANC-1 and MIA CaPa-2 cells. Due to a longer treatment time for PANC-1 and MIA CaPa-2 cells with nobiletin (14 days), we used lower concentrations of nobiletin (10, 20, 40 μM) for sphere formation assay. As shown in Figure 1 (D–E), compared with the control group, nobiletin significantly decreased the size and number of PANC-1 and MIA CaPa-2 spheres in a dose-dependent manner.

Effects of Nobiletin on Invasion and Migration of PANC-1 Cells

The effects of nobiletin on migration of PANC-1 cells was determined by wound-healing assays. Treating with nobiletin at 10-80 μM for 24 hours and 48 hours produced little growth inhibition of PANC-1 cells (cell viable inhibition rate was under 20%). These findings suggest that if wound-healing assays were performed within 48 hours, the effects of nobiletin at 10-80 μM would not be due to cytotoxicity, but instead due to inhibition of cell migration. Thus, we selected 40 and 80 μM for wound-healing assays. As shown in Figure 2 (A–B), treatment with different concentrations of nobiletin for 24 hours and 48 hours, significantly reduced PANC-1 cells moving into the wound. Moreover, we used a transwell assay with and without Matrigel to determine the effects of nobiletin on cell migration and invasion. After treatment with nobiletin for 24 hours, the number of cells that had migrated and invaded is shown in Figure 2 (C–E). Nobiletin reduced the migration and invasion abilities of PANC-1 cells in a dose-dependent manner. In addition, we investigated the effect of nobiletin on the levels of MMP-2 in PANC-1 cells. As shown in Figure 2 (F–G), treatment with nobiletin decreased the protein levels of MMP-2. These results indicate that inhibition of migration and invasion in

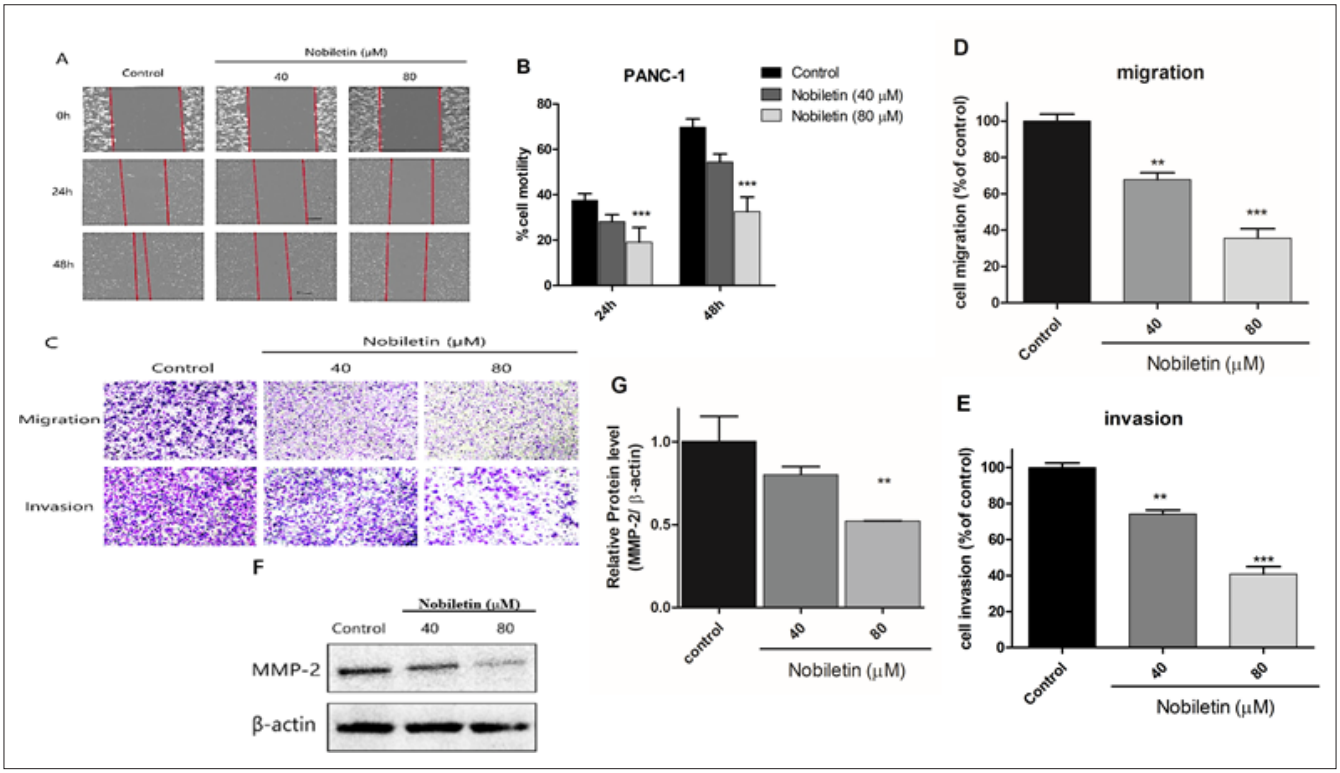

Figure 2. Effect of nobiletin on invasion and migration in PANC-1cells. (A) Cells were wounded and then treated with different concentrations of nobiletin for 24 hours and 48 hours. (B) The migration distance of PANC-1 cells is shown in the graph. Data shown are mean ± SEM, $n = 4$; $^{***}P < 0.001$ compared with the control group. (C) Transwell analysis of PANC-1 cells' ability for migration and invasion. Cells were treated with nobiletin for 24 hours before the number of migratory cells (D) and invasive cells (E) were determined. (F-G) PANC-1 cells were treated with nobiletin for 24 hours and the levels of MMP-2 were determined by Western blot. Data shown are mean ± SEM, $n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

PANC-1 cells was associated with downregulation of MMP-2 expression.

Effect of Nobiletin on the Levels of P-mTOR and P-STAT3 in PANC-1 Cells

Next, we investigated the possible regulatory mechanisms leading to inhibition of viability, migration, and invasion by nobiletin in PANC-1 cells. STAT3, a critical signaling molecule, has been shown to promote the growth, survival, migration, and attachment of cancer cells.[8,9,25] Therefore, we examined the inhibitory effects of nobiletin on STAT3 in PANC-1 cells. Western blot was performed to determine the expression levels of total and p-STAT3 protein. As shown in Figure 3(A and C), nobiletin significantly inhibited the expression of p-STAT3 in a dose-dependent manner. Furthermore, we determined the level of p-mTOR levels in PANC-1 cells. Hyperactivation of mTOR signaling promotes cell proliferation and metabolism that contribute to tumor initiation and progression,[26] Targeting mTOR may provide benefits for the treatment of pancreatic cancer.[26] In the present study, we found that nobiletin strongly decreased the level of p-mTOR at high concentration (Figure 3(A–B)). These results indicated that the effects of nobiletin in pancreatic cancer cells were associated with decreased activities of STAT3 and mTOR. In additional experiments, we determined the effect of knocking down STAT3 or mTOR on nobiletin-induced inhibition of PANC-1 cells. As shown in Figure 3(D), decreases in the expression levels of STAT3 and mTOR were confirmed by Western blotting. Treatment of the cells after STAT3 siRNA transfection with 80 or 120 μM nobiletin resulted in a reduced inhibitory effect on cell viability compared to the cells without STAT3 siRNA transfection (Figure 3(E); $P < 0.05$). Transfection with mTOR siRNA also significantly reduced the inhibitory effect of nobiletin on cell viability (Figure 3(E); $P < 0.05$).

Nobiletin Enhances the Anti-Cancer Effect of Gemcitabine on PANC-1 Cells

Since gemcitabine is commonly used in pancreatic cancer treatment, we sought to determine whether nobiletin could sensitize PANC-1 cells to gemcitabine. The viability of PANC-1 cells treated with nobiletin (20, 40, 80 μM) and gemcitabine (0.2 μM) was assessed after 72 hours treatment. As shown in Figure 4(A), compared with gemcitabine treatment alone, a stronger inhibitory effect on viability was observed with nobiletin and gemcitabine used together to treat PANC-1 cells. Moreover, we used the

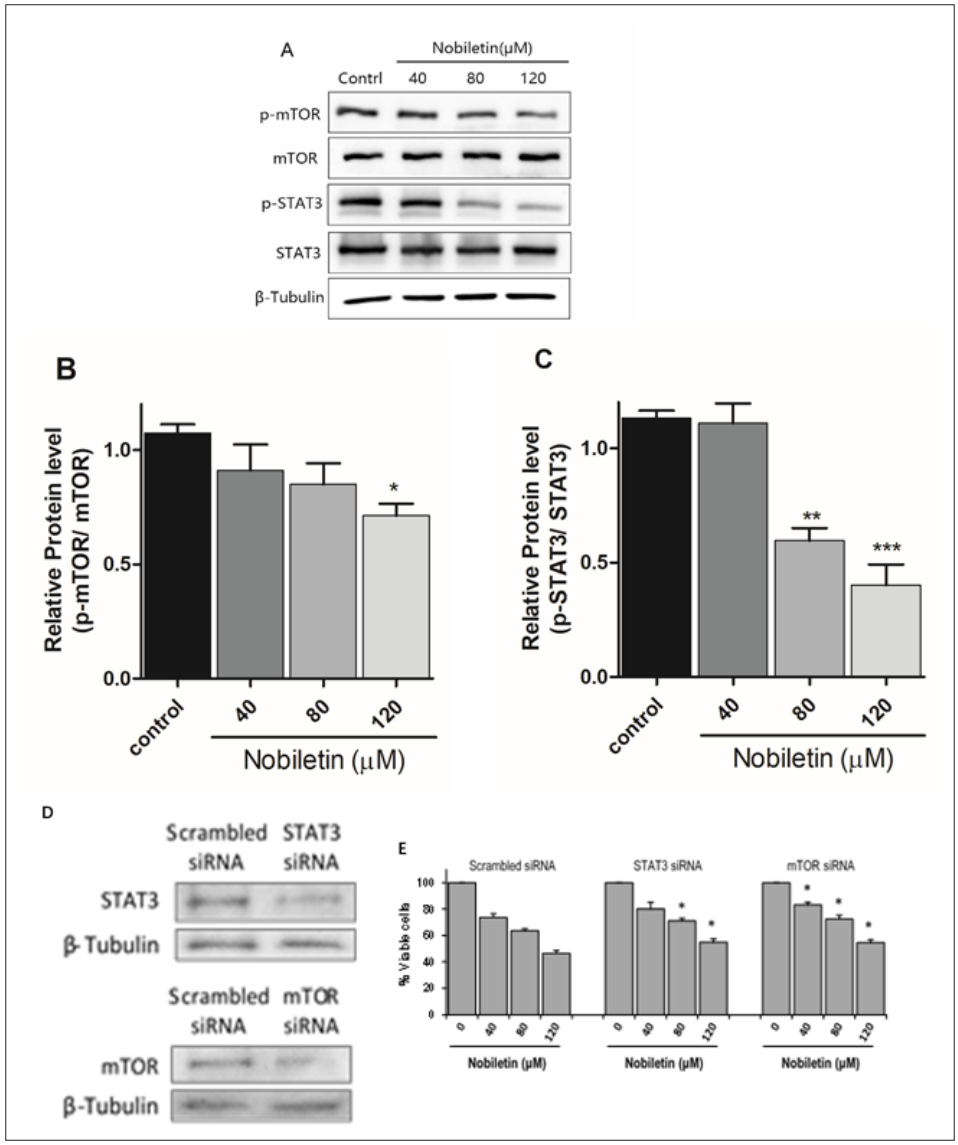

Figure 3. Effects of nobiletin on mTOR and STAT3 phosphorylation, and mTOR and STAT3 siRNA on nobiletin-induced growth inhibition of PANC-1 cells. PANC-1 cells were treated with nobiletin for 24 hours before p-mTOR/mTOR and p-STAT3/STAT3(A) levels were determined by Western blot. (B-C) The histograms show quantified results of protein levels. Data shown are mean ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. (D) PANC-1 cells were transfected with scrambled STAT3 or mTOR siRNA. The levels of STAT3 and mTOR were determined by Western blotting. (E) PANC-1 cells were transfected with scrambled STAT3 or mTOR siRNA and then treated with different concentrations of nobiletin. Cell viability was determined by MTT assay. Data shown are mean ± SEM, $n = 3$; $^{*}P < 0.05$ compared with control siRNA transfected cells treated with the same dose of nobiletin.

isobologram to analyze the synergistic effect of nobiletin and gemcitabine. The CI value is shown on the top of the column. PANC-1 cells treated with gemcitabine and nobiletin in combination showed strong synergy (CI = 0.25-0.46). We also determined the influence of knocking down STAT3 through siRNA on the synergistic effect of nobiletin combined with gemcitabine. As shown in Figure 4(B), transfection with STAT3 siRNA greatly reduced the synergy between nobiletin and gemcitabine (CI = 0.9-0.96). This result suggests that STAT3 is important for the synergistic interactions of gemcitabine and nobiletin. In addition, we selected a low concentration of gemcitabine (1 nM) combined with nobiletin (10, 20 μM) for treating PANC-1 cells in the sphere formation assay. As shown in Figure 4(C and D), the combination of nobiletin and gemcitabine decreased the size and number of PANC-1 spheres in a dose-dependent manner.

Effects of Nobiletin and Gemcitabine on the Levels of P-mTOR and P-STAT3

To further investigate the mechanism of nobiletin and gemcitabine in synergistically inhibiting the viability of PANC-1 cells, we determined the levels of p-mTOR and p-STAT3 in the cells by

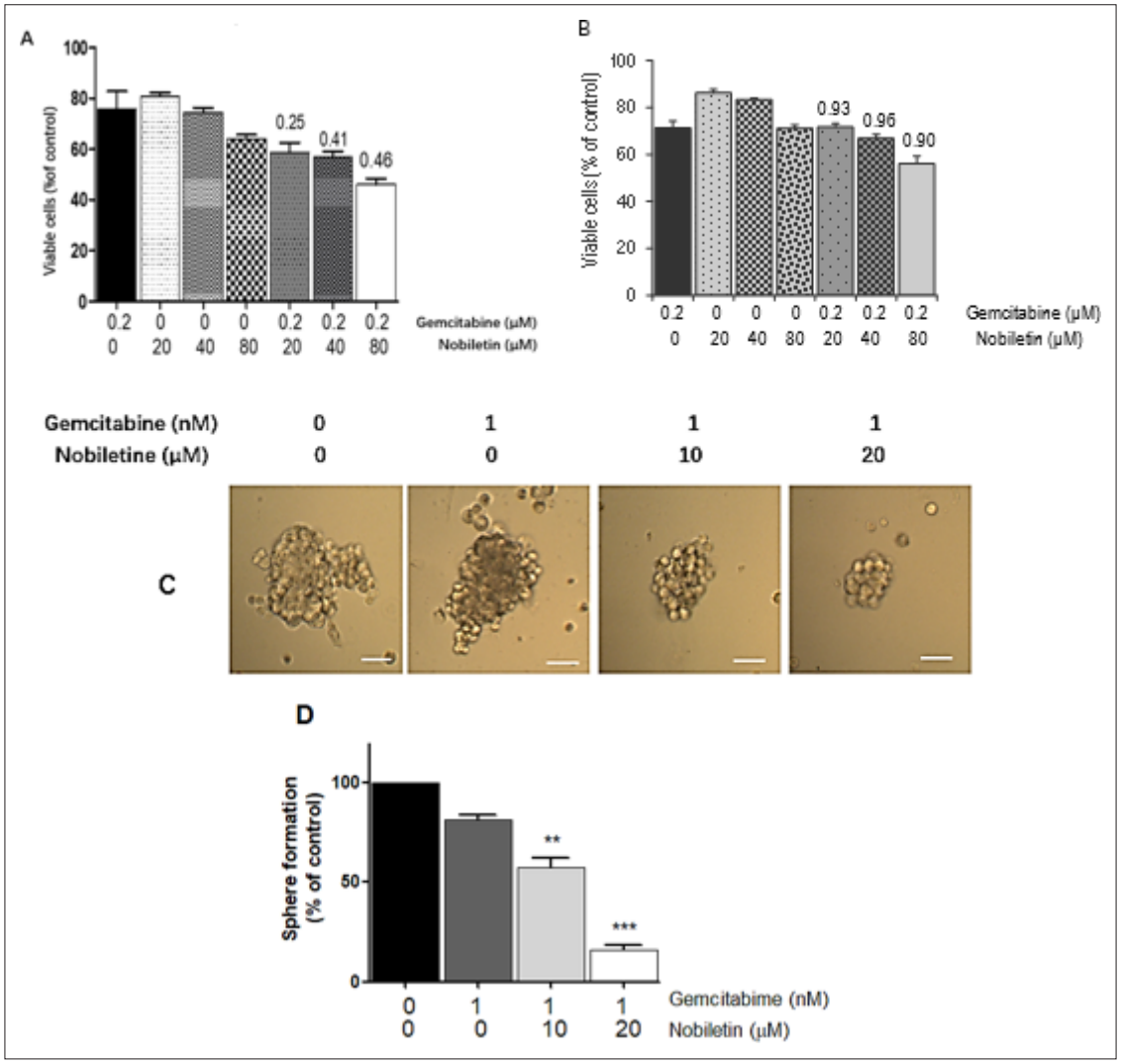

Figure 4. Nobiletin enhances the anti-cancer effect of gemcitabine in PANC-1 cells. Cell viability was assessed using MTT after treatment with different concentrations of nobiletin and/or gemcitabine for 72 hours in PANC-1 cells without transfection with STAT3 siRNA (A) and transfected with STAT3 siRNA (B). The combination index (CI） is shown on the top of the column for each combination. Effects of nobiletin and/or gemcitabine on sphere formation in PANC-1 cells. (C) Tumor spheres were captured under the microscope. (D) The number of sphere formations. Data shown are mean ± SEM, $n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with gemcitabine group.

Western blot. As shown in Figure 5, treatment with 0.2 μM gemcitabine had small effect on decreasing the levels of p-STAT3 and p-mTOR in PANC-1 cells. Combining gemcitabine with nobiletin in the treatment showed a stronger effect on decreasing the level of p-STAT3, but showed no inhibitory effect on level of p-mTOR in PANC-1 cells. These results thus provide evidence that the synergistic effect of gemcitabine and nobiletin on PANC-1 cells was associated with decrease in the levels of p-STAT3.

Discussion

We have been interested in developing effective combinations for the treatment of pancreatic cancer. In our continuing search for natural compounds that can enhance the efficacy of chemotherapeutic drugs, nobiletin was found to have a strong combined effect with gemcitabine. Therefore, we further evaluated the effects of nobiletin on migration, invasion and sphere formation in pancreatic cancer cells. We also investigated the mechanisms and molecular targets important for the synergistic effect of nobiletin and gemcitabine. The present study provides important information for our future studies on nobiletin analogs.

Nobiletin has been shown to inhibit proliferation and induce apoptosis in pancreatic cancer cells.[19] In a recent study, Jiang and colleagues found that nobiletin inhibited the growth and metastasis of pancreatic cancer MIA PaCa-2 cells via induction of autophagy and inhibition of the NF-κB signaling pathway.[16] Nobiletin inhibits migration and invasion in osteosarcoma and nasopharyngeal cancer cells.[27,28] In the present study, we found that nobiletin inhibited migration and invasion

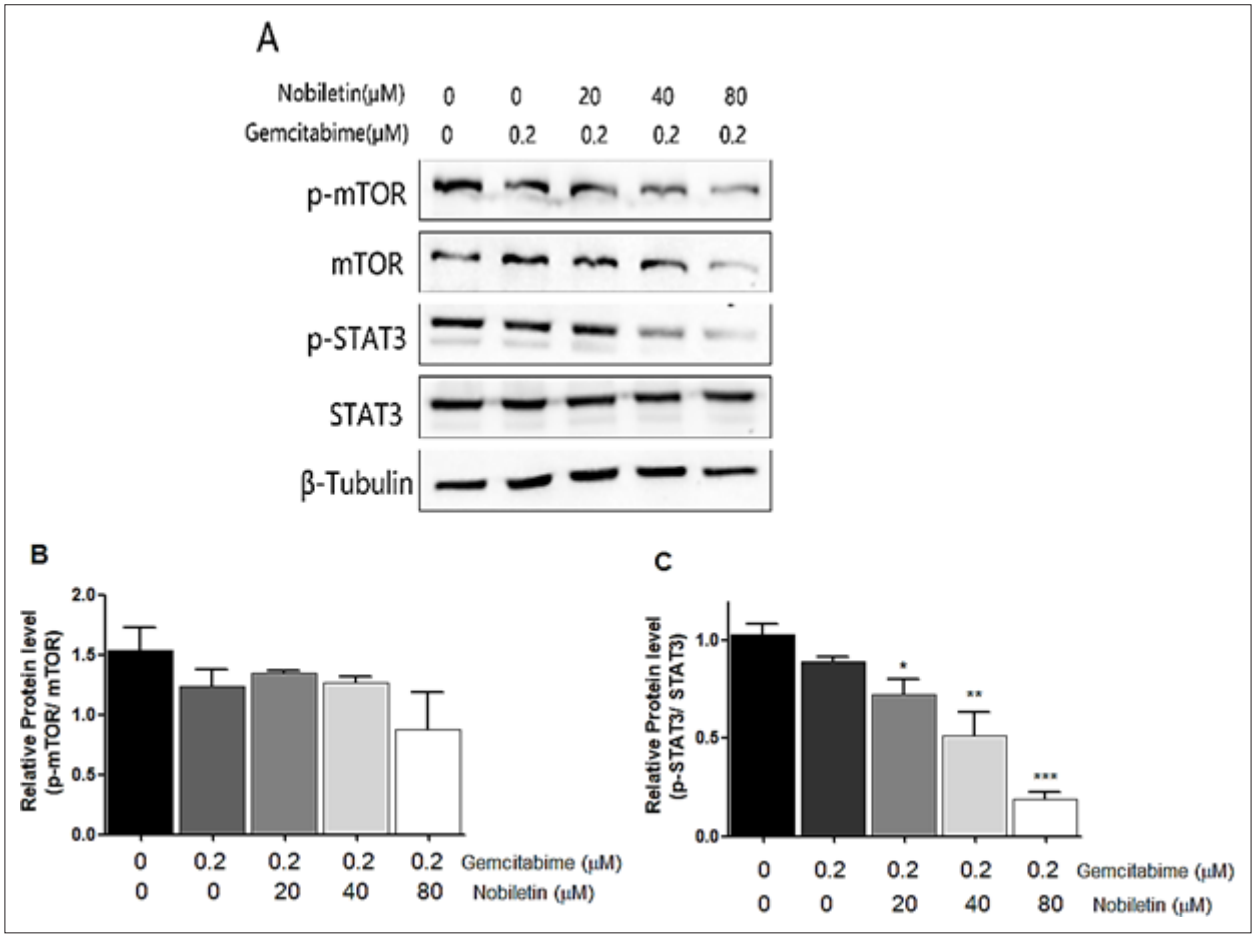

Figure 5. Effect of nobiletin and gemcitabine on the level of p-mTOR/mTOR, p-STAT3/STAT3 in PANC-1 cells. (A) PANC-1 cells were treated with nobiletin and gemcitabine for 24 hours, and then p-mTOR/mTOR and p-STAT3/STAT3 levels were determined by Western blot. (B-C) The histograms show quantified results of protein levels. Data shown are mean ± SEM, $n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with gemcitabine group.

of PANC-1 cells. To understand the molecular mechanism related to the effects of nobiletin on migration and invasion of pancreatic cancer cells, we examined the expression of proteins associated with migration and invasion. Matrix metalloproteinases (MMPs) play important roles in tumor invasion and metastasis. It is well established that inhibition of MMP gene expression or enzyme activity is an effective way to prevent cancer metastasis.[29,30] Pharmacological inhibitors of MMP have been used in clinical trial.[29] MMP-2 was most widely studied showing that both gain and loss-of function are associated with the invasive metastatic potential of cancer cells.[27,30] Our studies showed that nobiletin significantly inhibited the migration and invasion of PANC-1 cells, and these effects were associated with down-regulating the expression of MMP-2.

To explore further the molecular mechanisms of nobiletin on inhibiting pancreatic cancer cells, we determined the effects of nobiletin on STAT3 and mTOR, which have been shown to play an important role in pancreatic cancer cell growth and metastasis.[31,32] STAT3 is well known to be activated by many trans-membrane growth factor receptors, cytosolic kinases, and the Janus kinases (JAK),[33,34] which suggest that targeting STAT3 could be a promising way to treat pancreatic cancer.[25,35] Previous studies have shown that nobiletin effectively inhibited the activation of STAT3.[18,36] However, the effect of nobiletin on the levels of STAT3 in PANC-1 cells has not been reported. In the present study, we found that nobiletin decreased the level of phosphorylated STAT3 in PANC-1 cells. Clinical study has shown that approximately 15% to 20% of human pancreatic cancers exhibit high levels of active phosphorylated mTOR, and these patients have significantly reduced survival.[26] Many studies indicate that inhibition of mTOR may provide benefits for the treatment of pancreatic cancer.[7,23,37] mTOR is one of the downstream proteins of EGFR, and both STAT3 and mTOR can effectively promote cell proliferation, invasion and metastasis in pancreatic cancer cells.[36,38,39] mTOR signaling is also involved in the development of resistance to anticancer therapies.[37] STAT3 and mTOR activation are also associated with inflammation in pancreatic cancer tissues and may serve as targets for inhibiting cancer-related

inflammation.[3,40] Therefore, we examined the effect of nobiletin on mTOR expression in PANC-1 cells, and the result showed that nobiletin had an inhibitory effect on mTOR. Our results suggest that the inhibitory effects of nobiletin on growth, migration and invasion of PANC-1 cells were associated with suppression of STAT3 and mTOR activation. Moreover, the results of our study demonstrated the knocking down of STAT3 and mTOR by siRNA decreased the inhibitory effect of nobiletin on PANC-1 cell viability. These results suggest that the inhibitory effect of nobiletin on PANC-1 cell viability is likely mediated by inhibition of STAT3 and mTOR phosphorylation. Future studies are needed to explore the mechanisms by which nobiletin inhibits STAT3 and mTOR phosphorylation.

Gemcitabine-based chemotherapy is a commonly used treatment for pancreatic cancer. However, chemotherapy with gemcitabine alone has limited success because of the development of drug resistance.[25] Therefore, there is an urgent need for strategies to enhance chemosensitivity to gemcitabine, which would improve the outcome of gemcitabine chemotherapy. Suppression of STAT3 was shown to either inhibit cancer cells or increase the efficacy of chemotherapeutic agents.[8,9,39,41] A growing body of evidence has suggested that the combination of different anticancer agents may produce enhanced efficacy in comparison with the effects produced by each individual agent alone.[33,42] In the present study, we investigated whether nobiletin could enhance the effect of gemcitabine on PANC-1 cells. Our results showed that nobiletin in combination with gemcitabine synergistically inhibited PANC-1 cell growth, and the combination strongly decreased the level of phosphorylated STAT3. Moreover, we found that knocking down STAT3 through siRNA decreased the synergistic effect of nobiletin and gemcitabine. This result suggests that STAT3 phosphorylation is important for the synergistic interaction between nobiletin and gemcitabine.

Conclusion

In summary, the results of the present study demonstrated that nobiletin had strong inhibitory effects on cell viability, sphere formation, migration and invasion of human pancreatic cancer cells. The results also demonstrated that nobiletin and gemcitabine in combination synergistically inhibited the viability of PANC-1 cells. Mechanistic studies indicate that the inhibition of migration and invasion in PANC-1 cells by nobiletin was associated with downregulation of MMP-2. The inhibitory effect of nobiletin on cell viability was associated with decreases in phosphorylated STAT3 and mTOR. Our study suggests that STAT3/pSTAT3 is important for the synergistic interaction between nobiletin and gemcitabine. The present study provides strong in vitro evidence for the anti-pancreatic cancer activity of nobiletin. Further studies using suitable animal models to examine the in vivo effects of nobiletin alone or in combination with gemcitabine are warranted.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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