Piperlongumine Suppresses Growth and Sensitizes Pancreatic Tumors to Gemcitabine in a Xenograft Mouse Model by Modulating the NF-kappa B Pathway

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Abstract

Pancreatic cancer is an aggressive malignancy, which generally respond poorly to chemotherapy. Hence, novel agents that are safe and effective are highly needed. The aim of this study was to investigate whether piperlongumine, a natural product isolated from the fruit of the pepper Piper longum, has any efficacy against human pancreatic cancer when used either alone or in combination with gemcitabine in vitro and in a xenograft mouse model. In vitro, piperlongumine inhibited the proliferation of pancreatic cancer cell lines, potentiated the apoptotic effects of gemcitabine, inhibited the constitutive and inducible activation of NF-κB, and suppressed the NF-κB–regulated expression of c-Myc, cyclin D1, Bcl-2, Bcl-xL, Survivin, XIAP, VEGF, and matrix metalloproteinase-9 (MMP-9). Furthermore, in an in vivo xenograft model, we found piperlongumine alone significantly suppressed tumor growth and enhanced the antitumor properties of gemcitabine. These results were consistent with the downregulation of NF-κB activity and its target genes, decreased proliferation (PCNA and Ki-67), decreased microvessel density (CD31), and increased apoptosis (TUNEL) in tumor remnants. Collectively, our results suggest that piperlongumine alone exhibits significant antitumor effects against human pancreatic cancer and it further enhances the therapeutic effects of gemcitabine, possibly through the modulation of NF-κB– and NF-κB–regulated gene products.

Introduction

Pancreatic cancer remains one of the most aggressive and lethal human malignancies having an alarmingly low 5-year survival rate, even with the best treatment available today, of less than 5% (1, 2). In the year 2013 in the United States alone, almost 45,220 men and women were diagnosed with pancreatic cancer and 38,460 died of disease (3). The poor prognosis of pancreatic cancer is mostly due to the lack of ability for early detection, the aggressive biologic behavior of the tumor and the insensitivity to chemotherapy (4). Gemcitabine, erlotinib, and abraxane are the only FDA-approved agents to treat pancreatic cancer. However, they produce a poor response rate and are associated with multiple adverse events and chemoresistance. Hence, there is an urgent need for novel strategies involving less toxic agents that can overcome the resistance and enhance the effect of chemotherapy (4). Gemcitabine, erlotinib, and abraxane are the only FDA-approved agents to treat pancreatic cancer. 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Figure 1. Piperlongumine suppresses the proliferation and potentiates the apoptotic effects of gemcitabine in pancreatic cancer cells in vitro. A, chemical structure of piperlongumine. B, cell viability assay. Three pancreatic cancer cells (BxPC-3, PANC-1, and AsPC-1) were grown in the absence or presence of increasing concentration of piperlongumine for 24, 48, and 72 hours and cell viability was measured by CCK-8 assay. Data shown are representative of at least three independent experiments. C, cell apoptosis assay. Cells were pretreated with piperlongumine or left untreated as control for 24 hours before incubating with or without gemcitabine for 48 hours, and then apoptosis was quantitated using a Cell Death Detection ELISA kit. D, cells were viewed under a laser scanning confocal microscopy. Representative photographs were taken from Annexin V/PI-stained pancreatic cancer cells under certain treatment. E, Western blotting analysis. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis to monitor the expression of procaspase-3 and PARP. β-Actin was detected as protein loading control. Gem = Gemcitabine, PL = Piperlongumine, PL + Gem = Piperlongumine + Gemcitabine. *, P < 0.05 relative to control; #, P < 0.05 relative to single agent.
In the present report, we investigated whether piperlongumine alone could inhibit the growth of human pancreatic cancer cells both in vitro and in a xenograft mouse model, and whether piperlongumine could sensitize human pancreatic cancer to gemcitabine. Our observations indicate for the first time that piperlongumine inhibited the in vitro proliferation of various pancreatic cancer cells, potentiated gemcitabine-induced apoptosis, and significantly enhanced the antitumor effects of gemcitabine in human xenograft pancreatic cancer model, which was correlated with inhibition of NF-κB activity and downregulation of NF-κB downstream gene products.

Materials and Methods

Materials

Piperlongumine was purchased from Sigma-Aldrich, Inc. Gemcitabine (Gemzar) was purchased from Eli Lilly. Antibodies used in this study included Antibodies against Survivin, procaspase-3, c-Myc, Bcl-2, Bcl-xL, VEGF, PCNA, and XIAP (Santa Cruz Biotechnology). Antibodies against Ki-67 and CD31 (Abcam Inc.), and Antibodies against cyclin D1, PARP (Cell Signaling Technology). The methodology has been described previously (41). In brief, the transfections were performed following the instructions outlined by the manufacturer. After transfection, cells were treated as designed before being harvested for the ATCC short tandem repeat profiling.

Cell viability assay

The viability of treated cells was determined by using the Cell Counting Kit-8 (CCK-8) Kit (Dojindo Laboratories) following the instructions outlined by the manufacturer and as previously described by us (40). Briefly, cells were plated at a density of 3 to 5 × 10^4 cells per well with 200 μL of medium in 96-well microtiter plates. After treatment, CCK-8 solution (10 μL) was added to each well and the plates were incubated at 37°C for 90 minutes. The absorbance of the cell suspension was measured with a microplate reader at a wavelength of 450 nm. The highest concentration of piperlongumine and gemcitabine as single agents and in combination do not interfere with the CCK-8 assay reagents, in the absence of cells (data not shown). Medium containing 10% CCK-8 served as a control.

Apoptosis assay

Apoptosis was evaluated by using the Cell Death Detection ELISA Kit following the instructions outlined by the manufacturer. Cells were also visualized under a laser scanning confocal microscope (LSM-510, Carl Zeiss Jena GmbH) to detect apoptosis.

Western blot analysis

To determine the levels of protein expression, cell lysates were prepared and subjected to Western blot analysis as described previously (40). Briefly, cells were washed twice in PBS, sonicated in RIPA buffer and homogenized. Debris was removed by centrifugation at 12,000 × g at 4°C for 10 minutes and protein concentration was determined using the BCA Protein assay according to the manufacturer’s instructions. Samples containing equal amounts of protein (50 μg) were separated by electrophoresis on 10% or 15% polyacrylamide SDS gels (100 V for 1 to 2 hours) and transferred to polyvinylidene difluoride membranes by electroblotting (100 V for 1 hour at 4°C). The running time and voltage as well as transfer time and voltage may require some optimization depending on the circumstances. The membranes were then blocked by incubating with 5% skim milk in TBST buffer (TBS plus 0.1% Tween 20) for 2 hours and incubated with the appropriate primary antibody with gentle agitation overnight at 4°C. The membranes were then washed several times and incubated with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody for 1 hour at room temperature. The membranes were incubated with appropriate secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature. Proteins were visualized using an enhanced chemiluminescence system and captured on X-ray films. β-Actin was simultaneously determined as a loading control.

Plasmids and transfections

The methodology has been described previously (41). In brief, BxPC-3 cells were seeded in 6-well plates and incubated for 24 hours at 37°C. The cells were then transfected with NF-κB p65 cDNA, control empty vector (pcDNA3.0), NF-κB p65 siRNA and control siRNA, respectively, using Lipofectamine 2000 (Invitrogen). The transfections were performed following the instructions outlined by the manufacturer. After transfection, cells were treated as designed before being harvested for the following studies.

Xenograft mouse model

Animal studies were in accordance with the institutional guide for the care and use of laboratory animals. Our experimental protocol was reviewed and approved by the Animal Care and Use Committee of Harbin Medical University (Harbin). Male nude BALB/c mice (4–6-weeks-old) were obtained from Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Tumors were established by subcutaneous injection of 5 × 10^6 BxPC-3 cells into the flanks of mice. Tumor size was measured...
every 3 days with calipers and the volume was estimated according to the formula: \( V = \left( \frac{r}{6} \right) \times \left( \text{larger diameter} \right) \times \left( \text{smaller diameter} \right)^2 \). When tumors reached around 100 mm\(^3\), the mice were randomly assigned to four groups (each group had 8 mice): (i) control (DMSO was dissolved in 200 \( \mu \)L PBS, once daily by i.p. injection); (ii) piperlongumine (10 mg/kg, once daily by i.p. injection); (iii) gemcitabine (100 mg/kg, twice weekly by i.p. injection); and (iv) piperlongumine and gemcitabine, following the same schedule of individual drugs. The doses of piperlongumine and gemcitabine selected for this experiment were based on preliminary experiments and previous studies (26, 34, 42). The mice were closely monitored for 24 days, then euthanized, and the tumors were removed. Each tumor was divided into two pieces: one fixed in 10% buffered formalin, and the other kept at \(-80^\circ\text{C}\) for further analysis.

**PCNA and Ki-67 immunohistochemistry**

The methodology has been described previously (40). In brief, paraffin-embedded tissue sections (5 \( \mu \)m) were immunostained with an anti-PCNA or anti-Ki-67 Ab. The number of PCNA or Ki-67–positive cells was counted in randomly selected ten microscopic fields at \( \times 400 \) magnification.

**Quantification of apoptosis in tumor sections**

The methodology has been described previously (40). In brief, paraffin-embedded tissue sections (5 \( \mu \)m) were stained with the TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) agent (Roche), and the number of TUNEL-positive cells was counted in randomly selected ten microscopic fields at \( \times 400 \) magnification.

**Tumor microvessel density**

The methodology has been described previously (43). In brief, tumor sections prepared from tumors 4 weeks after treatment were immunostained with anti-CD31 Ab. The number of microvessels was counted in randomly selected ten microscopic fields at \( \times 400 \) magnification, and the microvessel density was recorded.

**Immunohistochemical analysis for cyclin D1, MMP-9, and VEGF in tumor samples**

The expression of cyclin D1, MMP-9, and VEGF were evaluated by immunohistochemical analysis as described previously (9).

**NF-κB DNA-binding activity assay**

The NF-κB DNA-binding activity was determined using the Trans-Am NF-κB/p65 ELISA Kit following the instructions outlined by the manufacturer and as described previously (4). Briefly, nuclear and cytosolic fractions were prepared from cells by using the Nuclear Extraction Kit following the instructions outlined by the manufacturer. Briefly, nuclear lysate protein from each group was added to a 96-well plate with an immobilized oligonucleotide containing the specific consensus sequence (5'–GGGAG–TTTC–3') for NF-κB/p65 binding. Following incubation for 1 hour at room temperature to facilitate the binding, a primary antibody specific for NF-κB was added to each well, followed by a HRP-conjugated secondary antibody. The absorbance was taken at 450 nm using an ELISA plate reader.

**Statistical analysis**

Data are represented as mean values \( \pm \) SD. Statistical comparisons were made with a one-way ANOVA followed by the Dunnett \( t \)-test for multiple comparisons. A \( P \) value of less than 0.05 was considered significant.

**Results**

The purpose of this study was, first, to determine whether piperlongumine, a natural product isolated from the fruit of the pepper Piper longum has potential in the treatment of pancreatic cancer when used either alone or in combination with gemcitabine and, second, to explore the molecular mechanism(s) behind these effects. For this, we used three different well-characterized human pancreatic cancer cell lines. To monitor tumor growth in vivo, one of these cell lines, BxPC-3 was subcutaneously injected and used in a mouse xenograft model.

**Piperlongumine suppresses the proliferation and potentiates the apoptotic effects of gemcitabine in pancreatic cancer cells in vitro**

To investigate the effect of piperlongumine on the proliferation of human pancreatic cancer cells, we used BxPC-3, PANC-1, and AsPC-1 cells, all of which exhibit differential resistance pattern to conventional chemotherapeutic drugs. The cells were assessed for viability using a CCK-8 assay following exposure to increasing concentrations of piperlongumine (0–40 \( \mu \)mol/L) for 24, 48, or 72 hours. As shown in Fig. 1B, piperlongumine suppressed the growth of all three human pancreatic cancer cells in a dose- and time-dependent manner.

To determine whether piperlongumine could enhance the apoptotic effects, we treated pancreatic cancer cells with piperlongumine combined with gemcitabine and then examined these cells with a histone DNA ELISA assay. Our results showed that treatment with either piperlongumine or gemcitabine alone induced apoptosis in all three cell lines tested. Relative to single agents, piperlongumine pretreatment followed by gemcitabine treatment led to significantly higher apoptosis in the cancer cells (Fig. 1C). Laser scanning confocal microscopy confirmed the increased apoptotic effect as shown in the representative photographs (Fig. 1D).

Next, BxPC-3, PANC-1, and AsPC-1 cells were treated with piperlongumine combined with gemcitabine and then caspase-3 activation and PARP cleavage were examined by Western blot analysis. Our data showed that piperlongumine and gemcitabine alone induced caspase-3 activation and PARP cleavage in all three pancreatic cancer cells and the combination was more effective (Fig. 1E).

**Piperlongumine inhibits constitutive as well as gemcitabine-induced NF-κB activation in pancreatic cancer cells**

Our results indicate that piperlongumine can potentiate gemcitabine-induced apoptosis in pancreatic cancer cells. How the effects were potentiated was investigated. Because NF-κB has been associated with both proliferation and apoptotic resistance, we next examined the effects of piperlongumine, gemcitabine, and the combination on NF-κB activity using an ELISA-based DNA-binding assay. Consistent with previous studies (9, 40, 43, 44), gemcitabine alone was able to induce NF-κB DNA-binding activity (Fig. 2B). In addition, our results showed that NF-κB p65 siRNA transfection significantly inhibited gemcitabine-induced NF-κB DNA-binding activity whereas NF-κB p65 CDNA transfection enhanced activation of NF-κB DNA-binding activity stimulated by gemcitabine.
Figure 2. Piperlongumine inhibits NF-κB and NF-κB–regulated gene products. A, NF-κB DNA-binding activity assay. Cells were treated with indicated concentration of piperlongumine for 4 hours, and then nuclear extracts were prepared. NF-κB DNA-binding activity on respective nuclear extracts was detected by ELISA-linked DNA-binding assay. B, cells were pretreated with piperlongumine or left untreated as control for 24 hours before incubating with or without gemcitabine for 48 hours, and then nuclear extracts were prepared and tested for NF-κB DNA-binding activity by ELISA-linked DNA-binding assay. C, Western blotting analysis. Cells were pretreated with piperlongumine or left untreated as control for 24 hours before incubating with or without gemcitabine for 48 hours, and then whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis to monitor c-Myc, Cyclin-D1, Survivin, Bcl-2, Bcl-XL, XIAP, MMP-9, and VEGF by Western blot. β-Actin was detected as protein loading control. The immunoblots shown here are representative of at least three independent experiments with similar results. Gem = Gemcitabine, PL = Piperlongumine, PL + Gem = Piperlongumine + Gemcitabine. *, P < 0.05 relative to control; †, P < 0.05 relative to gemcitabine.
As shown in Fig. 2A, we further found that treatment with piperlongumine caused a concentration-dependent decrease in NF-κB DNA-binding activity in all three pancreatic cancer cells. More importantly, piperlongumine was also found to significantly suppress gemcitabine- or p65 cDNA transfection–induced activation of NF-κB DNA-binding activity (Fig. 2B and 3A), which indicate that piperlongumine is a potent modulator of constitutive as well as inducible NF-κB activation in pancreatic cancer cells.

Numerous proteins, including c-Myc, cyclin D1, Bcl-2, Bcl-xL, Survivin, and XIAP, are regulated by NF-κB at the transcriptional level and associated with chemoresistance. To investigate whether piperlongumine exerts its effects through modulation of NF-κB–regulated proteins, we examined the effects of piperlongumine, gemcitabine, and the combination on the expression of NF-κB–regulated gene products implicated in cell proliferation (c-myc and cyclin D1), antiapoptosis (Bcl-2, Bcl-xL, Survivin, and XIAP), angiogenesis (VEGF), and metastasis (MMP-9). Our results showed that piperlongumine, alone or in combination with gemcitabine, significantly decreased the expression of all of these molecules compared with vehicle in all three pancreatic cancer cells (Fig. 2C). All these results suggest that potentiation of gemcitabine-induced apoptosis by piperlongumine in pancreatic cancer cells may be mechanistically linked with inactivation of NF-κB and its downstream genes.

The apoptosis-inducing and -chemosensitizing properties of piperlongumine are mediated by the NF-κB pathway.

NF-κB p65 cDNA or siRNA was transfected into BxPC-3 cells, and then treated with piperlongumine and gemcitabine according to the above-mentioned protocol of pretreatment with piperlongumine followed by gemcitabine treatment. Our results showed that overexpression of NF-κB by transfection of p65 cDNA enhanced the expression and activation of NF-κB p65 and protected cells from apoptosis in piperlongumine-treated and untreated BxPC-3 cells (Fig. 3A and C). Conversely, suppression of NF-κB by transfection of p65 siRNA abrogated NF-κB DNA-binding activity and augmented gemcitabine-induced apoptosis (Fig. 3B and D). Furthermore, we found that piperlongumine plus p65 siRNA exerted a more robust suppression of NF-κB DNA-binding activity and an augmented effect on the induction of apoptosis (Fig. 3B and D). Piperlongumine plus gemcitabine were more effective than gemcitabine alone at inhibiting NF-κB activation and inducing apoptosis in p65 cDNA-transfected BxPC-3 cells (Fig. 3A and C). These data provide direct evidence for the functional importance of NF-κB in piperlongumine-induced apoptosis and chemosensitization.

Piperlongumine potentiates the antitumor effects of gemcitabine in xenograft nude mice model.

On the basis of these results, we decided to examine the therapeutic potential of piperlongumine and gemcitabine, alone
highly significant tumor reduction in the piperlongumine and gemcitabine group was not only reducing the tumor burden. The tumor volume in the combination with gemcitabine was more effective in comparison with piperlongumine alone group; and piperlongumine in combination with gemcitabine compared with control. As shown in Fig. 5G and H, piperlongumine or gemcitabine treatment caused a significant increase in the percentage of TUNEL-positive cells in the group of piperlongumine in combination with gemcitabine compared with controls or single-agent–treated groups. Together, our results suggest that piperlongumine can augment the antitumor effects of gemcitabine by further suppressing tumor angiogenesis and promoting tumor cell apoptosis.

Piperlongumine inhibits NF-κB DNA-binding activity and NF-κB–regulated gene products in pancreatic tumor tissues

We also evaluated the effect of piperlongumine or gemcitabine on NF-κB levels in pancreatic tumor tissues. Our results clearly show that the constitutive activation of NF-κB in pancreatic cancer tissue was downregulated by piperlongumine, but most importantly, the gemcitabine treatment resulted in the induction of NF-κB DNA-binding activity similar to those observed in our in vitro studies, and this induction was abrogated in tumors treated with piperlongumine (Fig. 6A).

NF-κB is known to regulate the expression of genes involved in proliferation (c-myc and cyclin D1), antiapoptosis (Bcl-2, Bcl-XL, Survivin, and XIAP), invasion/metastasis (MMP-9), and angiogenesis (VEGF). Whether piperlongumine and gemcitabine can modulate the expression of these gene products in pancreatic tumor tissues was also determined by Western blot analysis. As shown in Fig. 6B, piperlongumine alone or in combination with gemcitabine, significantly decreased the expression of all of these
molecules compared with vehicle in the xenografts. The Western blot results also showed that piperlongumine, alone or in combination with gemcitabine, significantly decreased the expression of procaspase-3 and induced cleavage of PARP-1 in mouse tumors (Fig. 6B).

Whether modulation of cyclin-D1, MMP-9, and VEGF can also be detected by immunohistochemical methods. As shown in Fig. 6C, immunohistochemical analysis revealed that piperlongumine reduced the expression of all these proteins whether alone or with gemcitabine, which is consistent with the data obtained from western blotting experiments.

Collectively, these data suggest that modulation of NF-κB activity and NF-κB–regulated gene products is, at least, one of the molecular mechanisms by which piperlongumine exerts its effects in vitro, which is consistent with our in vitro findings.

**Discussion**

Pancreatic cancer is among the most aggressive human malignancies with a 5-year survival rate of less than 5% despite the best treatment available today. Therefore, novel therapeutic agents that are nontoxic and highly effective to improve the current standard of care of this disease are highly desirable. The present study was designed to investigate whether piperlongumine, a natural product isolated from the fruit of the pepper Piper longum, can potentiate the therapeutic effect of gemcitabine against human pancreatic cancer. In all three pancreatic cancer cell lines, piperlongumine inhibited proliferation, potentiated the cytotoxic effect of gemcitabine, and inhibited constitutive and gemcitabine-induced activation of NF-κB. In a pancreatic cancer xenograft model, piperlongumine augmented the antitumor activity of gemcitabine; this effect was associated with the downregulation of NF-κB activation and NF-κB–regulated gene products, decreased tumor cell proliferation (PCNA and Ki-67), decreased microvessel density (CD31), and increased tumor cell apoptosis (TUNEL).

We observed that piperlongumine can suppress the proliferation of all three pancreatic cancer cell lines in a dose- and time-dependent manner. This is the first report to describe the growth-inhibitory effects of piperlongumine in pancreatic cancer cells. The detailed molecular mechanism(s) by which piperlongumine exerts its inhibitory effects on cell proliferation were also investigated. We found that piperlongumine downregulated the expression of cell proliferative (c-myc and cyclin D1) and antiapoptotic (Bcl-2, Bcl-Xl, XIAP, and survivin) gene products. We also found that piperlongumine downregulated the constitutive activation of NF-κB in pancreatic cancer cell lines. These results on downregulation of NF-κB activation by piperlongumine are consistent with previous reports that piperlongumine inhibited activation of NF-κB in other cancer cell lines (30, 39) and with reports that resveratrol (45), genistein (46), dihydroartemisinin (44), zyflamend (47), γ-tocotrienol (48), curcumin (49), shikonin (9), and 3,3'-Diindolylmethane (50), all present within piperlongumine, are associated with NF-κB inactivation.

Specifically, our results also demonstrated that piperlongumine has a potential to enhance the effect of gemcitabine in human pancreatic cancer cells in vitro. We found that this effect may be mediated through the downregulation of cell survival proteins such as Bcl-2, Bcl-XL, XIAP, and survivin by piperlongumine. Interestingly, we also observed for the first time that piperlongumine suppressed both constitutive and gemcitabine-induced...
Piperlongumine inhibits NF-κB and NF-κB-regulated gene products in BxPC-3 xenograft tumors. Nuclear extracts and total protein extracts were prepared from subcutaneous tumors 24 days after treatment with vehicle, gemcitabine, piperlongumine, or piperlongumine + gemcitabine. A, NF-κB DNA-binding activity assay. NF-κB DNA-binding activity on respective nuclear extracts was detected by ELISA-linked DNA-binding assay. B, Western blotting analysis. Whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blot analysis to monitor the expression of c-Myc, Cyclin-D1, Survivin, Bcl-2, Bcl-XL, XIAP, VEGF, MMP-9, procaspase-3, and PARP. β-Actin was detected as protein loading control. The immunoblots shown here are representative of at least three independent experiments with similar results. C, immunohistochemical analysis. The expressions of Cyclin-D1, VEGF, and MMP-9 were examined by using immunohistochemical staining. D, proposed mechanisms of piperlongumine action in augmenting the efficacy of gemcitabine in pancreatic cancer. Gem = Gemcitabine, PL = Piperlongumine, PL + Gem = Piperlongumine + Gemcitabine; *, P < 0.05 relative to control; †, P < 0.05 relative to gemcitabine.
NF-κB activation. NF-κB has been linked with chemoresistance, so it is very likely that the inactivation of NF-κB by piperlongumine is mechanistically associated with sensitization of pancreatic cancer cells to gemcitabine-induced killing. Furthermore, using NF-κB cDNA transfection and NF-κB p65 siRNA approach, we demonstrated that NF-κB activation performs a crucial function in protecting cells against the cytotoxic effects of gemcitabine. This notion is further strengthened by the fact that NF-κB signaling in piperlongumine-mediated cytotoxic synergism with gemcitabine in pancreatic cancer cells. We also noticed decreased proliferation and increased apoptosis as documented by increased TUNEL staining within tumors, in tumor remnants of combination treatment with piperlongumine and gemcitabine. Furthermore, we demonstrated that the combination of piperlongumine and gemcitabine potentiates the antitumor effects of gemcitabine in vivo in a subcutaneous pancreatic cancer model. Our results clearly showed that the i.p. administration of piperlongumine alone preserved the growth of human pancreatic tumors in nude mice. Moreover, piperlongumine was very well tolerated by the animals. We also found that the piperlongumine-gemcitabine combination had a much more effective and potent antitumor effect than either agent alone. When examined for the mechanism by which piperlongumine exerts its effects against human pancreatic cancer, we noticed decreased proliferation as documented by PCNA and Ki-67 immunostaining, decreased microvesSEL density as documented by CD31 immunostaining, and increased apoptosis as documented by increased TUNEL staining within tumors, in tumor remnants of combination treatment with piperlongumine and gemcitabine. Furthermore, we demonstrated that piperlongumine inhibited constitutive and gemcitabine-induced NF-κB activation. Piperlongumine, alone or in combination with gemcitabine, significantly downregulated the expression of NF-κB-regulated gene products such as c-Myc, Cyclin D1, Bcl-2, Bcl-xl, Survivin, and XIAP in pancreatic tumor samples. These results are in good agreement with our molecular studies in vitro, indicating that the suppression of NF-κB and its downstream targets is, at least, one of the molecular mechanisms by which piperlongumine may exert its effects against human pancreatic cancer (Fig. 6D). In summary, the results from our cell-based and in vivo studies suggest for the first time that piperlongumine has great potential for the treatment of pancreatic cancer, and it can further potentiate the efficacy of gemcitabine through the suppression of NF-κB and its downstream regulated genes that are implicated in proliferation, angiogenesis, invasion, metastasis, and chemoresistance. On the basis of our findings, further in-depth studies, including clinical trials, are required to fully appreciate the value of piperlongumine as an anticancer agent in pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y. Wang, X. Wu, B. Sun

Development of methodology: X. Wu, H. Jiang, S. Pan, B. Sun

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zhou, H. Jiang, S. Pan, B. Sun

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, X. Wu, Y. Zhou, B. Sun

Writing, review, and/or revision of the manuscript: Y. Wang, B. Sun

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhou, S. Pan

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