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Synergistic effects of piperlongumine and gemcitabine against KRAS mutant lung cancer

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Abstract

Objective: To determine the combined efficacy of piperlongumine and gemcitabine for treatment of *KRAS* mutant lung cancer.

Methods: The cell growth inhibition of piperlongumine, gemcitabine, and piperlongumine plus gemcitabine was measured by Cell Counting Kit-8 assay and the combination index was calculated. In addition, the combined effects of piperlongumine and gemcitabine on cell apoptosis, reactive oxygen species (ROS) contents, and microtubule-associated protein 1 light chain 3B (LC3B) expression were examined.

Results: Piperlongumine increased ROS contents and LC3B-II expression. Following the combined treatment with piperlongumine and 10 mM N-acetyl-L-cysteine (NAC), intracellular ROS and cell viability returned to normal levels, and the expression of LC3B-II decreased to the predose level. Gemcitabine also induced cell apoptosis, increased ROS contents, and LC3B-II expression. The combination of piperlongumine with gemcitabine exhibited a synergetic anticancer activity with the combination index <1. The combined application of gemcitabine and piperlongumine yielded synergistic effects on cell apoptosis, but failed to synergistically increase ROS levels and LC3B-II expression.

Conclusion: Combination therapy with piperlongumine and gemcitabine is a promising treatment option for *KRAS* mutant lung cancer.

Keywords

piperlongumine, gemcitabine, lung cancer, reactive oxygen species, autophagy

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Introduction

Lung cancer is the leading cause of cancer-related death and most commonly diagnosed cancer worldwide, with 1.8 million deaths and 2.1 million new occurrences estimated in 2018.[1] Lung adenocarcinoma is the most common histologic subtype. The *KRAS* mutation rate in patients with lung adenocarcinoma is close to 30%.[2] The prevalence of *KRAS* mutation is relatively high in the Caucasian population when compared with Asian ethnicity.[2,3] Autophagy is involved in regulation of cell growth, lipid metabolism, and mitochondrial function of *KRAS*-driven lung cancer cells.[4] Microtubule-associated protein 1 light chain 3B (LC3B) is essential for autophagosome development and therefore is used to monitor autophagic activity. Activation of the autophagic process causes the conversion of LC3B-I to LC3B-II.[5]

Reactive oxygen species (ROS) are involved in cancer initiation, promotion, and progression.[6,7] Common ROS include superoxide, hydrogen peroxide, and hydroxyl radical.[6] Low ROS levels are shown to regulate cell growth, mitosis, angiogenesis, and cell survival in cancers, whereas high ROS contents exert cytotoxic effects on cancer cells by inducing cell cycle arrest and cell death.[8]

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Piperlongumine, also known as piplartine, is an amide alkaloid isolated from Piper longum L (long piper) commonly grown in southern India and southeastern Asia. Structurally, piperlongumine contains 7,8-unsaturated bonds and 2 Michael acceptors.[9,10] Piperlongumine is reported to increase ROS levels in osteosarcoma, breast, bladder, colon, pancreatic, and lung cancer cells.[10] Piperlongumine selectively kills cancer cells including osteosarcoma, pancreatic cancer, breast cancer, leukemia, and lung cancer, but does not have antiproliferative effects on normal cells.[10,11] In contrast to the results in cancer cells, piperlongumine did not cause an increase in ROS levels in normal cells.[12] Piperlongumine is found to exert its anticancer activity through ROS-mediated signaling pathways.[13]

Gemcitabine is a deoxycytidine analog that has been used as a common chemotherapeutic agent for non-small cell lung cancer.[14] Increased ROS contents induced by gemcitabine serve as one of the mechanisms for its cytotoxic effects.[15] Piperlongumine is also shown to induce cell death via a ROS-mediated mechanism.[13] Therefore, the current study hypothesized that induction of ROS upon gemcitabine treatment may enhance toxic activity of piperlongumine on cancer cells. Piperlongumine has been reported to preferentially kill senescent cells induced by ectopic expression of the oncogene *RAS*.[16] Since no targeted therapy has been clinically approved for the treatment of *KRAS* mutant lung cancer, it is meaningful to explore new treatment strategies. In the present study, antitumor effects of piperlongumine in combination with gemcitabine on *KRAS* mutant lung cancer cells were examined. The underlying mechanisms were investigated further.

Methods

Cell lines and cell culture

KRAS mutant lung adenocarcinoma A549 cells (cell bank of Chinese Academy of Science, Shanghai, China) were cultured at 37ºC with 5% carbon dioxide in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY).

Cytotoxicity assay

The cytotoxicity of piperlongumine (Selleck Chemicals, Houston, TX) or gemcitabine (Selleck Chemicals) was quantified by Cell Counting Kit–8 (CCK-8) assay. A549 cells were seeded in 96-well plates at a density of 3000 cells/well. After incubation with gemcitabine or piperlongumine, 10 μL of CCK-8 solution was added to each well. The optical density (OD) value of absorbance at 450 nm was determined using a Multiskan Go Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The cell growth inhibition rate of piperlongumine or gemcitabine was calculated as follows: (1 − OD450 of treated cells/OD450 of untreated cells) × 100%.

Detection of cell apoptosis

An annexin V/propidium iodide (PI) double-staining kit (BD Biosciences, San Jose, CA) was used to measure cell apoptosis. A549 cells were divided into 4 groups: the control group (did not treat with gemcitabine or piperlongumine), the piperlongumine group (treated with 8 μM piperlongumine), the gemcitabine group (treated with 5 μM gemcitabine), and the combination group (treated with 5 μM gemcitabine and 8 μM piperlongumine). A total of 1×10^5 cells were harvested via centrifugation and suspended in 100 μL of binding buffer. The cells were then incubated with 5 μL fluorescein isothiocyanate-labeled enhanced annexin V and 5 μL PI in the dark at room temperature for 15 minutes. The cell apoptosis rate was measured using BD FACS Calibur flow cytometer (BD Biosciences). Apoptotic cells were defined as annexin V-positive cells.

Western blot analysis

Total protein was extracted from A549 cells using radio immunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China). Equal quantities of protein (30–60 μg) were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel using a mini-gel apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). The separated proteins were transferred to polyvinylidene difluoride membranes, which were then blocked with 5% nonfat dry milk in Tris-buffered saline with Tween-20 (TBST) for 1.5 hours at room temperature. The membranes were subsequently incubated overnight at 4ºC with rabbit anti-poly-ADP-ribose polymerase (PARP) polyclonal antibodies (1:100; Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-LC3B polyclonal antibodies (1:1000; Novus Biologicals, Littleton, CO), and anti-α-tubulin monoclonal antibodies (1:3000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After washing with TBST, membranes were incubated with secondary horseradish peroxidase–coupled antibodies. Chemiluminescent signals were visualized using an enhanced chemiluminescence kit.

Measurement of intracellular ROS contents

Following treatment with piperlongumine or gemcitabine, A549 cells were collected and subsequently incubated with 10 μM 2′-,7′-dichloro-fluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology) at 37°C for 30 minutes. The cells were washed with serum free medium. The fluorescent intensity was determined using BD FACS Calibur flow cytometer (BD Biosciences).

Statistical analysis

All statistical analyses were performed using SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL). Normally distributed data are presented as the mean ± SD. Between-group differences were analyzed using the Student *t* test. Multigroup comparisons were assessed by one-way analysis of variance. A p value < 0.05 was considered statistically significant.

Results

Piperlongumine induces cell death through a ROS-mediated mechanism

Treatment of *KRAS* mutant lung cancer cells with 8 μM piperlongumine significantly increased ROS contents (Figure 1[A]). Addition of 10 mM N-L-acetylcysteine (NAC) reversed the increase in ROS levels and cytotoxic effects caused by piperlongumine treatment (Figure 1[A] and [B]).

NAC reverses the effects of piperlongumine on LC3B expression

The expression of LC3B-II was significantly increased after A549 cells were treated with 8 μM piperlongumine ($P < 0.05$). Notably, 10 mM NAC abolished the effects of piperlongumine on LC3B-II expression (Figure 2).

Effects of gemcitabine on ROS levels and LC3B expression

After treatment with 5 μM gemcitabine, ROS levels and LC3B-II expression of A549 cells were significantly increased (Figures 3 and 4).

Effects of piperlongumine and gemcitabine on ROS levels and LC3B expression

The combined application of 5 μM gemcitabine and 8 μM piperlongumine did not exert synergistic effects on ROS contents and LC3B expression (Figures 3 and 4).

Cytotoxic effects of piperlongumine and gemcitabine on KRAS mutant lung cancer cells

The growth inhibition rates of 5 μM gemcitabine and 8 μM piperlongumine on A549 cells were 33.07±4.97% and 26.24±5.81%, respectively, whereas inhibition rates of cells treated with piperlongumine plus gemcitabine were significantly higher than those treated with either agent alone (50.49±5.26%; $p < 0.05$; Figure 5). The combination index value for gemcitabine (5 μM) plus piperlongumine (8 μM) was 0.69. The cell growth inhibition rates of 7.5 μM gemcitabine, 12 μM piperlongumine, and a combination of both were 38.17±5.04%, 37.00±9.05%, and 59.11±2.10%, respectively. The combination index value for gemcitabine (7.5 μM) plus piperlongumine (12 μM) was 0.70. These results indicated that combination of gemcitabine and piperlongumine yielded synergistic effects.

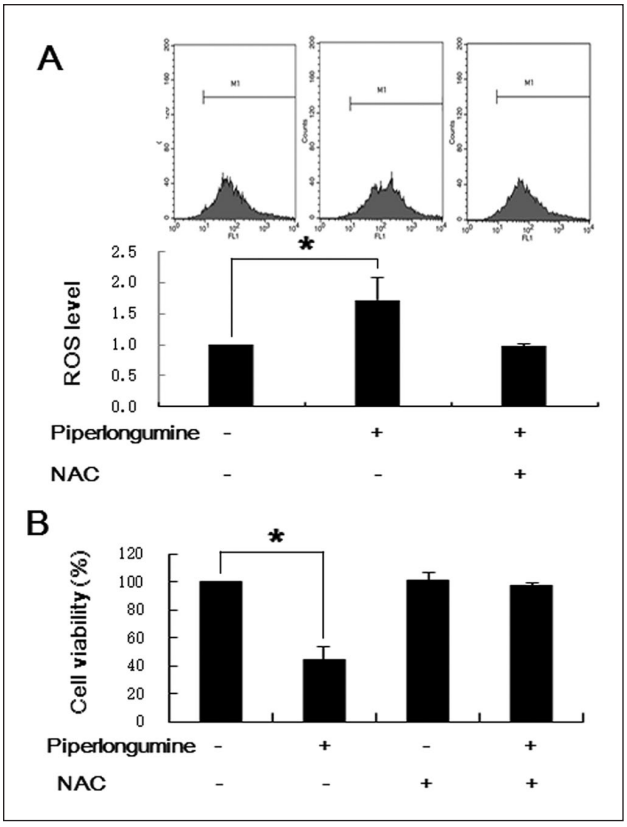

Figure 1. (A) and (B) Piperlongumine induces cell death through a reactive oxygen species (ROS)–mediated mechanism. NAC: N-L-acetylcysteine.

Piperlongumine and gemcitabine induce cell apoptosis

As shown in Figure 6(A) and (B), combination treatment induced a significant increase in the percentage of apoptotic cells: 9.63±1.77% for the control group, 12.03±0.57% for the gemcitabine group, 11.49±0.83% for the piperlongumine group, and 21.12±3.28% for the combination group. Western blots were performed to verify the combination effect of gemcitabine and piperlongumine. The protein level of cleaved PARP was significantly higher compared with the other 3 groups ($P < 0.05$; Figure 6[C]).

Discussion

KRAS has long been considered an undruggable target. AMG 510 has become the first drug to successfully target KRAS mutation.[17] In phase I trials, AMG 510 and MRTX849 have shown robust efficacy in patients with

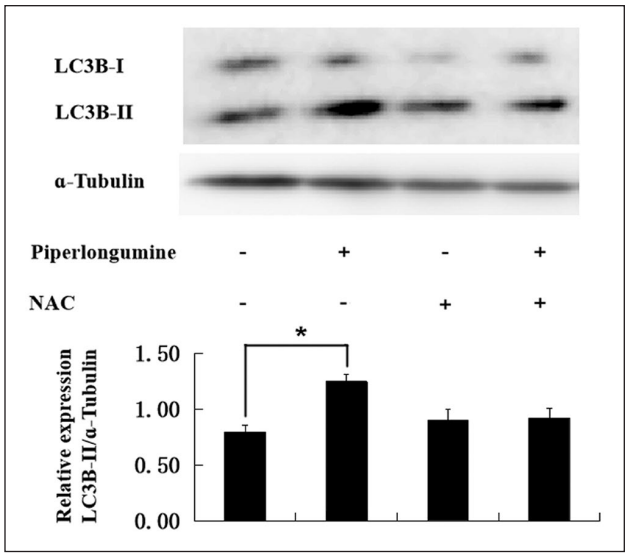

Figure 2. N-L-acetylcysteine (NAC) reverses upregulated expression of light chain 3B (LC3B)–II induced by piperlongumine.

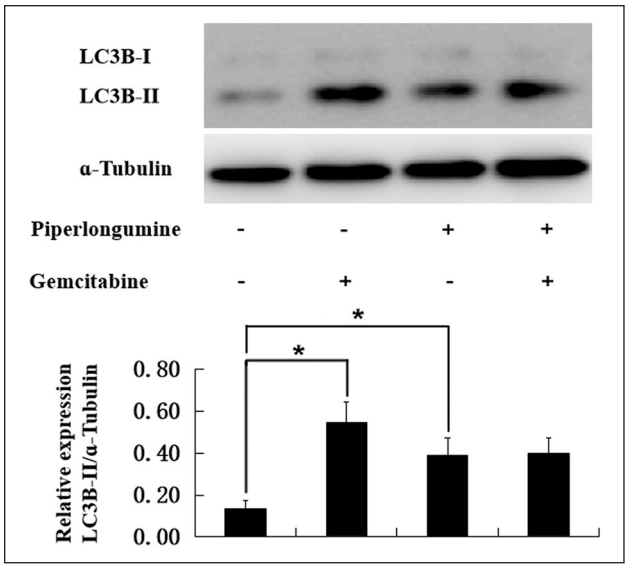

Figure 4. Effects of piperlongumine and gemcitabine on light chain 3B (LC3B) expression.

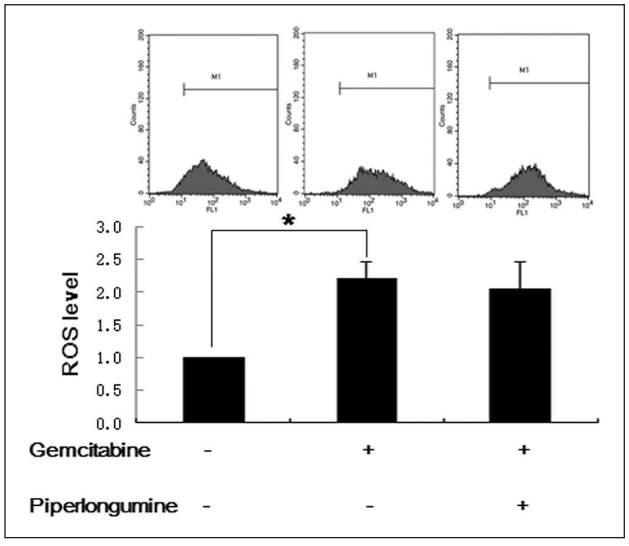

Figure 3. Effects of piperlongumine plus gemcitabine on reactive oxygen species (ROS) levels.

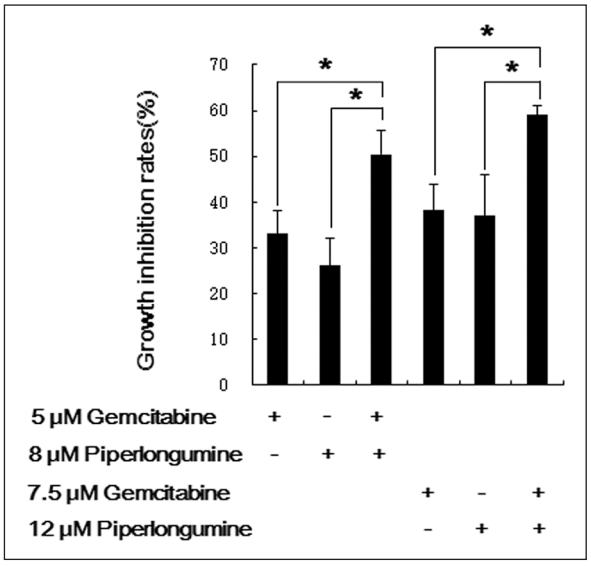

Figure 5. The combination of gemcitabine and piperlongumine synergistically increases growth inhibition against *KRAS*-mutant lung cancer cells.

KRAS mutation.[18] However, no targeted therapy has been approved for the treatment of *KRAS* mutant lung cancer. The present study investigated the combined efficacy of piperlongumine and gemcitabine for treatment of *KRAS* mutant lung cancer. The results indicated both piperlongumine and gemcitabine exerted cytotoxic effects, induced cell apoptosis, and increased ROS contents and LC3B-II expression. ROS scavenger NAC reversed cytotoxicity of piperlongumine. The combination of gemcitabine and piperlongumine yielded synergistic effects on cytotoxicity and cell apoptosis, but failed to synergistically increase ROS levels and LC3B-II expression.

Piperlongumine is found to selectively kill cancer cells. In the report by Raj et al.,[12] piperlongumine did not cause toxic effects in six different noncancerous cell types, whereas 13 cancer cell lines were killed by piperlongumine. In the present study, both piperlongumine and gemcitabine produced growth inhibition against A549 cells. The combination of piperlongumine with gemcitabine exhibited a synergetic anticancer activity with the combination index value <1.

Induction of cell apoptosis is one of the main mechanisms of many chemotherapeutic drugs.[19] Gemcitabine

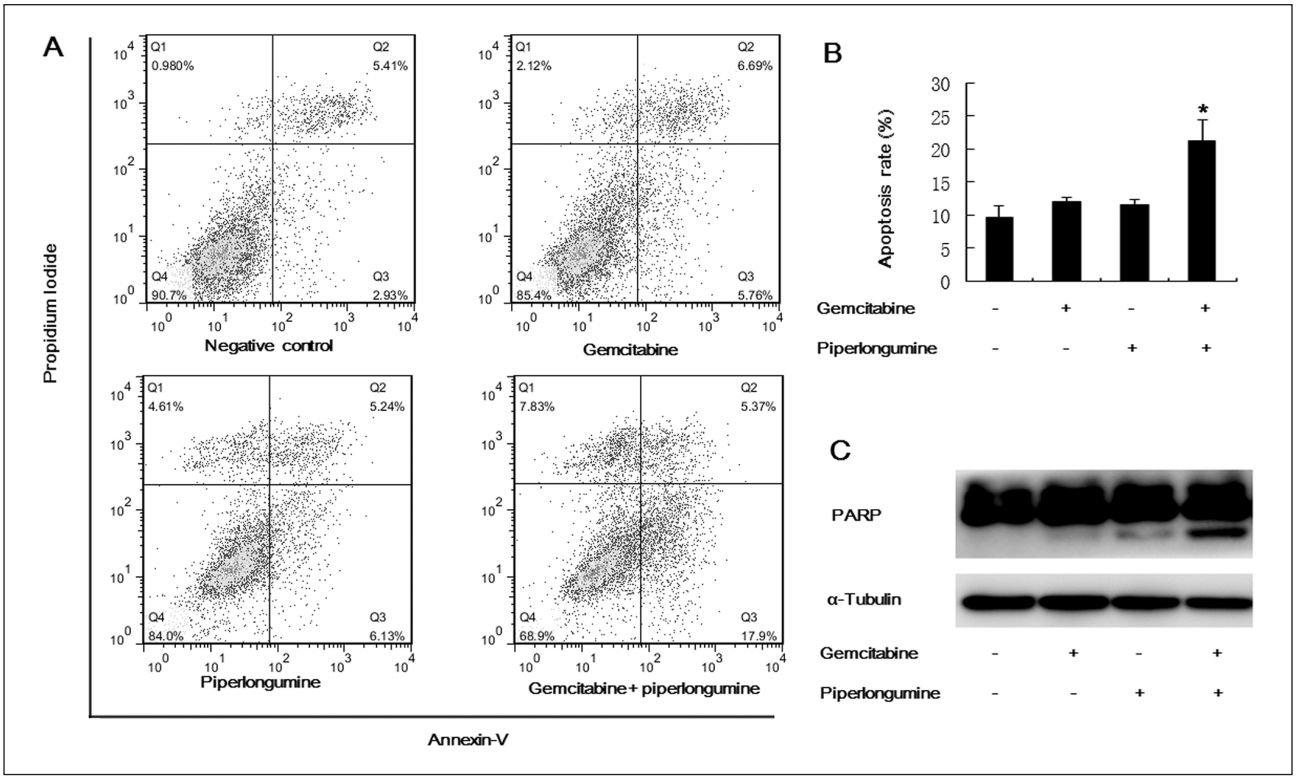

Figure 6. (A) to (C) Effects of piperlongumine and gemcitabine on cell apoptosis.

exerts antitumor effects primarily by inducing tumor cell apoptosis via blockage of DNA synthesis and repair.[20,21] Piperlongumine has also been found to selectively kill numerous cancer cell lines based largely on the induction of apoptosis.[22,23] In the present study, the combined application of piperlongumine and gemcitabine synergistically increased apoptotic rates of A549 cells and upregulated protein expression of cleaved PARP.

ROS play critical roles in regulating enzyme activity, signal transduction events, and cytokine production. Normal cells are inseparable from the role of ROS. However, deregulation of redox homeostasis is involved in cancer initiation and progression.[12,24] Cancer cells are more dependent upon the ROS stress response pathway than healthy cells, and as a consequence, are more vulnerable to drugs that cause further elevation of oxidative stress levels.[12,24] The mechanisms by which piperlongumine induces cancer-selective cell death have been found to be dependent on elevated ROS levels.[12,25] Similar results were observed in the current study, which showed that NAC reversed the increase in ROS levels and cytotoxic effects caused by piperlongumine treatment. Induction of ROS has been considered to be one of the mechanisms for antitumor activity of gemcitabine.[26] The present study also revealed that ROS levels of A549 cells were increased following treatment with gemcitabine. However, the combination of piperlongumine and gemcitabine did not synergistically increase ROS contents.

Apart from the induction of apoptosis, piperlongumine was shown to enhance autophagic activity.[9,27] The induction of autophagy by piperlongumine was found to be mediated through the upregulated expression of autophagic proteins (Beclin-1 and LC3B) in myeloid leukemia cells.[28] In the present study, the increased expression of LC3B-II was observed in piperlongumine-treated A549 cells. We further found that NAC abolished the effects of piperlongumine on LC3B-II expression. Wang et al.[29] also revealed that piperlongumine induced autophagy through ROS-induced stress response. It has been reported that gemcitabine treatment inhibits cell growth, induces apoptosis, and activates autophagy.[30] In the current study, LC3B-II expression was significantly increased on exposure to gemcitabine. However, the combination of piperlongumine and gemcitabine did not show synergistic effects on LC3B-II expression.

The combination of gemcitabine and piperlongumine synergistically increases cytotoxic effects and induces cell apoptosis. Combined therapy with piperlongumine and gemcitabine is a promising treatment option for *KRAS* mutant lung cancer. Further studies are needed to validate these results and assess the value of this strategy in a clinical setting.

Declaration of conflicting interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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