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Original Article

Piperine suppresses tumor growth and metastasis *in vitro* and *in vivo* in a 4T1 murine breast cancer model

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Aim: To investigate the effects of piperine, a major pungent alkaloid present in *Piper nigrum* and *Piper longum*, on the tumor growth and metastasis of mouse 4T1 mammary carcinoma *in vitro* and *in vivo*, and elucidate the underlying mechanisms.
Methods: Growth of 4T1 cells was assessed using MTT assay. Apoptosis and cell cycle of 4T1 cells were evaluated with flow cytometry, and the related proteins were examined using Western blotting. Real-time quantitative PCR was applied to detect the expression of matrix metalloproteinases (MMPs). A highly malignant, spontaneously metastasizing 4T1 mouse mammary carcinoma model was used to evaluate the *in vivo* antitumor activity. Piperine was injected into tumors every 3 d for 3 times.
Results: Piperine (35–280 μmol/L) inhibited the growth of 4T1 cells in time- and dose-dependent manners (the IC_{50} values were 105±1.08 and 78.52±1.06 μmol/L, respectively, at 48 and 72 h). Treatment of 4T1 cells with piperine (70–280 μmol/L) dose-dependently induced apoptosis of 4T1 cells, accompanying activation of caspase 3. The cells treated with piperine (140 and 280 μmol/L) significantly increased the percentage of cells in G_2/M phase with a reduction in the expression of cyclin B1. Piperine (140 and 280 μmol/L) significantly decreased the expression of MMP-9 and MMP-13, and inhibited 4T1 cell migration *in vitro*. Injection of piperine (2.5 and 5 mg/kg) dose-dependently suppressed the primary 4T1 tumor growth and injection of piperine (5 mg/kg) significantly inhibited the lung metastasis.
Conclusion: These results demonstrated that piperine is an effective antitumor compound *in vitro* and *in vivo*, and has the potential to be developed as a new anticancer drug.

Keywords: anticancer drug; piperine; 4T1 breast cancer; apoptosis; cell cycle; metastasis; MMP-9; MMP-13

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Introduction

Piperine (Figure 1) is a major pungent alkaloid present in black pepper (*Piper nigrum*) and long pepper (*Piper longum*). It exhibits a wide variety of biological effects. Piperine could inhibit both the drug transporter P-glycoprotein and the major drug-metabolizing enzyme CYP3A4[1, 2], so it has been used as a bioavailability enhancer with various structurally and therapeutically diverse drugs[3]. Piperine also had anti-inflammatory, anti-nociceptive, and anti-arthritic effects by inhibiting the expression of interleukin 6 (IL-6), matrix metalloproteinases 13 (MMP-13) and prostaglandin E2 (PGE2) in an arthritis animal model[4] or by inhibiting tumor necrosis factor-α (TNF-α) induced activation of NF-κB via blocking IκBα kinase activation[5]. Additionally, piperine also possessed anti-depression like activity, cognitive enhancing effect[6], a blood pressure-lowering effect[7], anti-oxidative, anti-apoptotic and chemo-protective ability in blastogenesis[8]. More of the diverse physiological effects of piperine have been reported in recent decades[9].

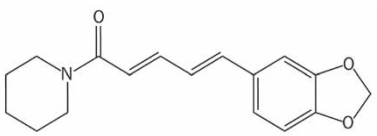

Figure 1. Chemical structure of piperine {1-[5-(1,3-benzodioxol-5-yl)-oxo-2,4-pentadienyl]piperidine}.

Concerning the antitumor effects, piperine has been reported to inhibit lung metastasis induced by B16F-10 mela-

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noma cells[10], and the anti-invasive effects of piperine on fibrosarcoma cells also have been demonstrated[11]. The therapeutic properties of piperine with other antitumor drugs were tested in various cell types[12, 13]. But there is little known about the anticancer activities of piperine on mammary cancer cells and the underlying mechanism.

The 4T1 tumors closely mimics human breast cancer in its anatomical site, immunogenicity and growth characteristics[14]. After sc inoculation in the abdominal mammary fat pad, the primary tumor grows into a nodule with the histology of a high-grade breast cancer and sheds spontaneous systemic metastases. Metastatic growth in the lungs is usually the main cause of death of mice. The pattern and histological appearance of such metastases are similar to what is seen in humans, therefore, this model is suitable for testing the effects of experimental therapies on metastatic disease. In the present study, we observed the effects of piperine on the tumor growth and metastasis of 4T1 breast cancer *in vitro* and *in vivo*. And the possible mechanism for the inhibitory effect of piperine on tumor cell growth and migration were investigated. The antitumor efficacy makes piperine a potential candidate for future cancer therapy.

Materials and methods

Materials

Piperine (molecular weight, 285.35) was purchased from Fluka (St Louis, MO, USA). A 50 mg/mL stock solution of piperine was prepared in DMSO and then further diluted in cell culture medium or PBS. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis, MO, USA). Annexin-V FITC Apoptosis Detection Kit was purchased from R&D systerms (Minneapolis, MN, USA). Reverse transcription enzymes and SYBR Green detection chemistry were obtained from Takara Bio Inc (Japan). Anti-Bcl-2 (sc-7382), anti-Bax (sc-526), anti-cyclin D1 (sc-8396), anti-cyclin B1 (sc-595), anti-β-actin (sc-4778) and IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-caspase 3 (1087-1) was purchased from Epitomics (Epitomics, CA, USA). All chemicals and reagents were of analytical grade.

Cell lines

4T1 mouse mammary carcinoma cells were a kind gift from Prof Yang-xin FU (Chicago University, Chicago, IL, USA). NIH3T3 (murine fibroblast cell line) cells were obtained from American Type Culture Collection (ATCC, Manassas, VT, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

Animals

Female BALB/c mice syngeneic to 4T1 cells, aged 5–6 weeks, were purchased from Joint Ventures Sipper BK Experimental Animal Co (Shanghai, China) and housed in a room maintained at constant temperature and humidity under 12-h light and darkness cycle and fed with regular autoclave chow diet with water. All animal usage was conducted according to protocols approved by the Zhejiang University Institutional Animal Care and Use Committee.

MTT assay

The effect of piperine on the growth of 4T1 and NIH3T3 cells was examined using a MTT assay. Cells were subcultured in 96-well plates at a density of 10^3 cells per well with or without piperine (35, 70, 100, 140, and 280 μmol/L) for 24 h, 48 h, or 72 h in a final volume of 200 μL. Then, the medium was removed and 20 μL of MTT (5 mg/mL in PBS) was added to the fresh medium. After 2 h incubation at 37 °C, 100 μL DMSO was added to each well and plates were agitated for 1 min. Spectrophotometric absorbance at 570 nm was measured. The percentage of viability was calculated as the following formula: (viable cells)%=(*OD* of drug-treated sample/*OD* of untreated sample)×100.

Apoptosis detection assay

After the treatment with piperine 0, 70, 140, and 280 μmol/L for 24 h, harvested cells were suspended in 100 μL binding buffer (1×) including 1 μL Annexin V-FITC and 10 μL PI for 15 min in dark at room temperature and then 400 μL binding buffer (1×) was added to each sample. The FITC and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10 000 events were acquired.

Cell cycle analysis

After the treatment with piperine 0, 70, 140, and 280 μmol/L for 24 h, 4T1 cells were collected, washed with cold PBS, fixed in cold 70% ethanol and stored at -20 °C overnight. Cells were then washed again with PBS before staining (100 μg/mL of RNase A, 25 μg/mL of propidium iodide and 0.1% of Triton X-100 in PBS), and incubated at 37 °C for 30 min. Cell cycle analysis was performed on flow cytometer.

Western blot analysis

4T1 cells were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) for 30 min on ice after the treatment with piperine 0, 70, 140, and 280 μmol/L for 24 h. Lysates were then centrifuged at 14 000×*g* for 10 min to remove insoluble material. Then a BCA kit (Pierce Biotechnology, Rockford, IL, USA) was used to determine the concentration of lysates. Cell extracts were separated by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat milk. The blot was probed with each antibody against Bcl-2, Bax, cyclin D1, cyclin B1, caspase 3, and β-actin. Then the blot was washed, exposed to horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Signals were detected as we previously described[15].

Wound healing assay

To study the effects of piperine on cell migration *in vitro*, a wound healing assay was performed following standard methods[16]. 4T1 cells were seeded in 6-well plates and grown

until 90% confluent. Cells were then serum starved for 24 h, and a linear wound was created in the confluent monolayer using a 200 μL pipette tip. Cells were then washed with PBS and diluted in RPMI-1640 containing 1% FBS with piperine at a concentration of 70 or 140 μmol/L. Images of the wound surfaces were recorded after 24 h.

RNA isolation and real-time quantitative PCR analysis

Total RNA from cells was extracted using TRIzol reagent in accordance with the manufacturer's instructions. Real-time quantitative PCR, using SYBR Green detection chemistry, was performed on a 7500 Real-Time PCR System (Applied Biosystems). Quantitative measurements were determined using the ΔΔCt method and expression of GAPDH was used as the internal control. The sequences of the primers are as shown in Table 1.

Evaluation of antitumor activity of piperine in 4T1 mammary carcinoma models

4T1 harvested from subconfluent cultures were washed once in serum-free medium and resuspended in PBS. Cells (5×10^5) in 0.1 mL PBS were implanted subcutaneously into the female BALB/c mice in the abdominal mammary fat pad. After 3 d of implantation, piperines (0, 2.5, and 5 mg/kg) were dissolved in 0.2% DMSO and injected into tumors every three days for three times. The tumor volume was measured every two or three days using the formula $V=0.5236\times d_1^2\times d_2$, where d_1 is the shortest diameter, and d_2 is the longest diameter. Tumor tissues for histology examination were collected on d 20, fixed in 10% formalin, and embedded in paraffin. Sections (5 μm thick) were prepared for hematoxylin–eosin staining.

Examination of lung metastases by colonogenic assay

Cells (5×10^5) in 0.1 mL PBS were implanted subcutaneously into the female BALB/c mice. After 3 d of implantation, piperine (0 and 5 mg/kg) were dissolved in 0.2% DMSO and injected into tumors every three days for three times. Five days after the last therapy, primary 4T1 tumors were eliminated from the experiments. For surgical excision of primary 4T1 tumors, mice were anesthetized, and tumors were resected with sterilized instruments. Wounds were closed with metallic clips. Mice in which primary tumors recurred at the site of the surgical excision were eliminated from the experiments. A colonogenic assay was used to evaluate metastases by 4T1 tumors as we previously described[15]. Briefly, lungs were collected and chopped before being dissociated in RPMI-1640 supplemented with 10% FBS containing 1.5 mg/mL collagenase type D (Sigma, St Louis, MO, USA) for 30 min in 37 °C shaking incubator at 178 r/min speed. Lungs were then plated at various dilutions in the RPMI-1640 supplemented with 10% FBS and 60 μmol/L 6-thioguanine. Individual colonies representing micrometastases were counted after 5–10 d.

Statistical analysis

All experiments were repeated two or three times. Data was described as the mean±SD, and statistical analysis was carried out using Student's *t*-test or the log-rank test (for survival analysis). The difference was considered statistically significant when the *P*-value was less than 0.05.

Results

Piperine inhibited the growth of 4T1 cells *in vitro*

Inhibitory effects of piperine on the growth of 4T1 cells were examined using the MTT assay. We also tested the effects of piperine on murine NIH3T3 fibroblast cell growth. Compared to the untreated cells (taken as 100% viable), different concentrations of piperine showed a time- and dose-dependent inhibition on 4T1 cell growth in the concentration range of 35~280 μmol/L (Figure 2A). Exposure of 4T1 cells to piperine at 140 μmol/L and 280 μmol/L for 24 h caused a marked decrease in the viability from 100% to 78% and 48% of untreated control levels, respectively. And 48 h of piperine treatment at 140 μmol/L and 280 μmol/L caused a decrease in the viability from 100% to 33% and 18%, respectively. The IC_{50} values for piperine were 105±1.08 μmol/L for 48 h, and 78.52±1.06 μmol/L for 72 h treatment (Figure 2C), suggesting a dose-dependent inhibition of growth, while piperine was not so toxic to NIH3T3 cells (Figure 2B), for the IC_{50} values was 232±1.15 μmol/L for 48 h treatment (Figure 2C).

Piperine induced apoptosis of 4T1 cells and increased the caspase 3 activity

In order to evaluate the mechanism of growth inhibition of 4T1 cells by piperine, apoptosis of 4T1 cells treated with various concentrations of piperine (0, 140, and 280 μmol/L) for 24 h was analyzed by Annexin V-FITC/PI double-labeled flow cytometry. The apoptosis rate was the sum of early apoptosis and late apoptosis. There was little binding of Annexin-V in untreated and 70 μmol/L piperine treated 4T1 cells (Figure 3A). But after treatment with piperine at 140 and 280 μmol/L for 24 h, the apoptosis rate was about 24.2%, 23.6%, respectively (Figure 3B).

Apoptosis is characterized by changes in the expression and activity of several apoptotic markers. The control and regula-

Table 1. Primer sequences used in real time PCR.

Gene	Forward (5′→3′)	Reverse (5′→3′)	Size (bp)
MMP-9	TGAGTTCCAGGGCACACCA	TGTCTGGAGATTCGACTTGAAGTC	91
MMP-13	GCCCATGAGCTTGGCCACTCC	GGGTCTTCATCGCCTGGACCATAA	158
MMP-14	TTCAGCCCCGAAGCCTGGCT	GAGGGCGCCTCATGGCCATC	178
GAPDH	TCTCCACTTTGCCACTGCAA	GAACGGATTTGGCCGTATTG	65

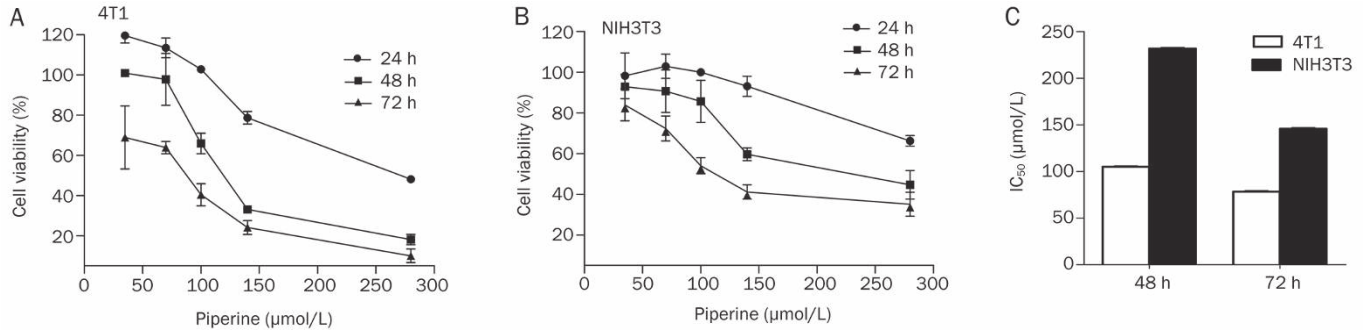

Figure 2. Inhibition of growth by piperine in 4T1 and NIH3T3 cells. 4T1 (A) and NIH3T3 (B) cells were treated with 0, 35, 70, 100, 140, and 280 μmol/L piperine for 24 h, 48 h, and 72 h. Cell viability was monitored by MTT assay. The percentage of viability was calculated as the following formula: (viable cells)%=(*OD* of drug-treated sample/*OD* of untreated sample)×100. Mean±SD. *n*=3. (C) The IC_{50} values for piperine on 4T1 and NIH3T3 cells.

tion of the apoptotic mitochondrial events occur through the members of the Bcl-2 family of proteins which can be pro-apoptotic (Bcl-10, Bax, *etc*) or anti-apoptotic (Bcl-2, Bcl-xL, *etc*). The expression levels of Bax and Bcl-2 are detected by Western blot. The results showed no change in the expression of Bcl-2 and Bax in 4T1 cells after piperine treatment (Figure 3C). Caspases are responsible for cell apoptosis. Therefore we determined the effect of piperine on the expression of caspases by Western blot analysis. The results indicated that caspase 3 activity was up-regulated after piperine treatment (Figure 3C).

Piperine caused the accumulation of the cells in G_2/M phase

After incubation with various concentrations of piperine (0, 70, 140, and 280 μmol/L) for 24 h, cell cycle distributions were analyzed by FCM. We found that with the dose of piperine increased, the percentage of cells in G_2/M phase was increased, and there were slight changes in G_0/G_1 phase cell population (Figure 4A). After incubation with 140 and 280 μmol/L piperine for 24 h, percentage of cells in G_2/M phase was increased to 7.3%, 15.5%, respectively (Figure 4B).

Piperine down-regulated the expression of cyclin B1

Given that cyclins are required for the cell cycle progression, the protein levels of cyclin D1 and cyclin B1 in 4T1 cells were detected by Western blot. The result showed that piperine treatment down-regulated the expression of cyclin B1 in a dose-dependent manner, with maximum suppression observed at 280 μmol/L (Figure 4C), while there was no change of cyclin D1 expression after piperine treatment with various concentrations.

Piperine inhibited the migration ability of 4T1 cells *in vitro*

We investigated the effects of piperine on the migration properties of 4T1 cells *in vitro* by wound healing assay. Cells were treated by piperine at a concentration of 0, 70, and 140 μmol/L, and the results showed that piperine inhibited the migration of 4T1 cells in a dose-dependent manner (Figure 5A).

Piperine decreased the mRNA expression of MMP-9 and 13

Matrix metalloproteinases (MMPs), comprise a family of zinc-containing endopeptidases that share common structural domains. These proteins have the capacity to degrade extracellular matrix (ECM) components, as well as alter their biological functions. Therefore, we examined the mRNA expression of MMP-9, 13, and 14 in 4T1 cells by real-time quantitative PCR after piperine treatment. The mRNA expression of MMP-9 and 13 in 4T1 cells, but not MMP-14, were decreased in a dose-dependent manner (Figure 5B).

Piperine suppressed 4T1 tumor growth *in vivo*

To test the antitumor effect of piperine, female BALB/c mice were inoculated sc with 5×10^5 4T1 cells. Piperines (0, 2.5, and 5 mg/kg) were injected intratumorally every three days for three times. The result showed that piperine exhibited a significant inhibition of tumor growth especially at a high dose (5 mg/kg) (Figure 6A). To determine whether the regulation of apoptotic proteins and cell cycle-related regulatory proteins was also involved in the inhibition of tumor growth by piperine *in vivo*, the tumors were harvested from each group and the expression level of these proteins were analyzed by Western blot. As shown in Figure 6B, the level of active caspase 3 was increased and cyclin B1 was reduced in tumors from the mice received piperine therapy, which was similar to the effect of piperine *in vitro*. Histopathological analysis of the tumor tissues excised from the control mice showed groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei and binucleation. Several degrees of cellular and nuclear pleomorphism were seen. In the tumors excised from the mice treated with piperine, extensive areas of coagulative necrosis were observed (Figure 6C).

Piperine also inhibited the lung metastasis of 4T1 tumors

4T1 cells can metastasize to various organs, such as lung, as early as d 10 after inoculation of 1×10^5 4T1 tumor cells[17]. To study the potential inhibitory effect of piperine on metastasis, we used a colonogenic assay to evaluate the metastases in the lung. We detected less metastasis in the lungs of the mice that had been given 5 mg/kg piperine treatment compared to the lungs of the control mice (Figure 7). The results indicated that piperine not only suppressed the local primary tumor growth,

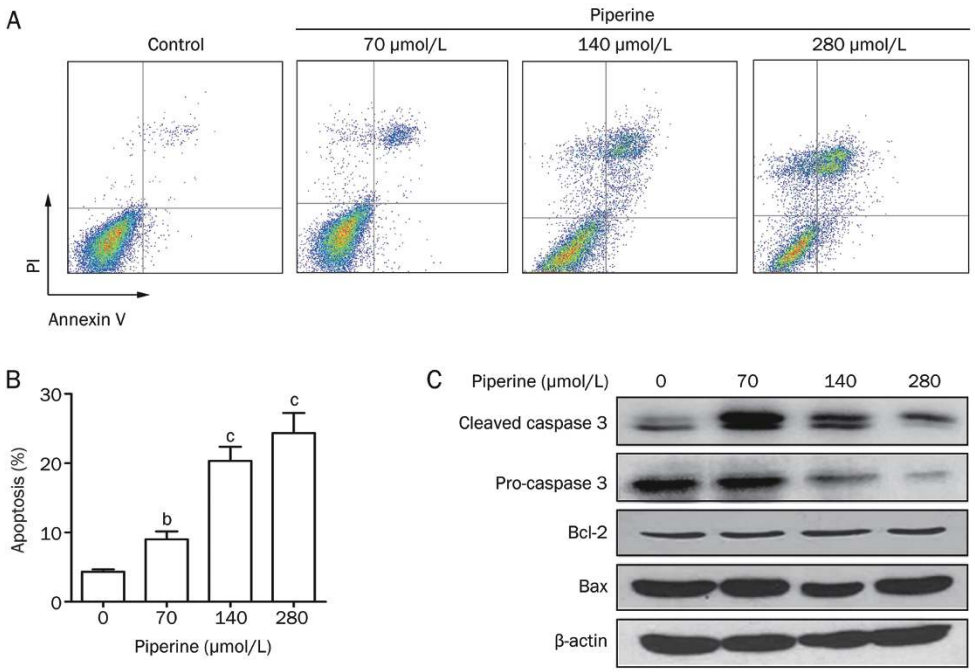

Figure 3. Piperine induced the apoptosis in 4T1 cells with the up-regulation of caspase 3. (A) 4T1 cells were treated with piperine (0, 70, 140, and 280 μmol/L) for 24 h. The induction of apoptosis was detected by Annexin V-FITC/PI double staining assay. (B) The apoptotic cell death was quantified as Annexin V$^+$ (both PI-negative and PI-positive) cells. Mean±SD. n=3. ^bP<0.05, ^cP<0.01. (C) Western blot analysis of caspase 3, Bcl-2, and Bax in 4T1 cells treated with or without piperine. Similar results were obtained in four independent experiments.

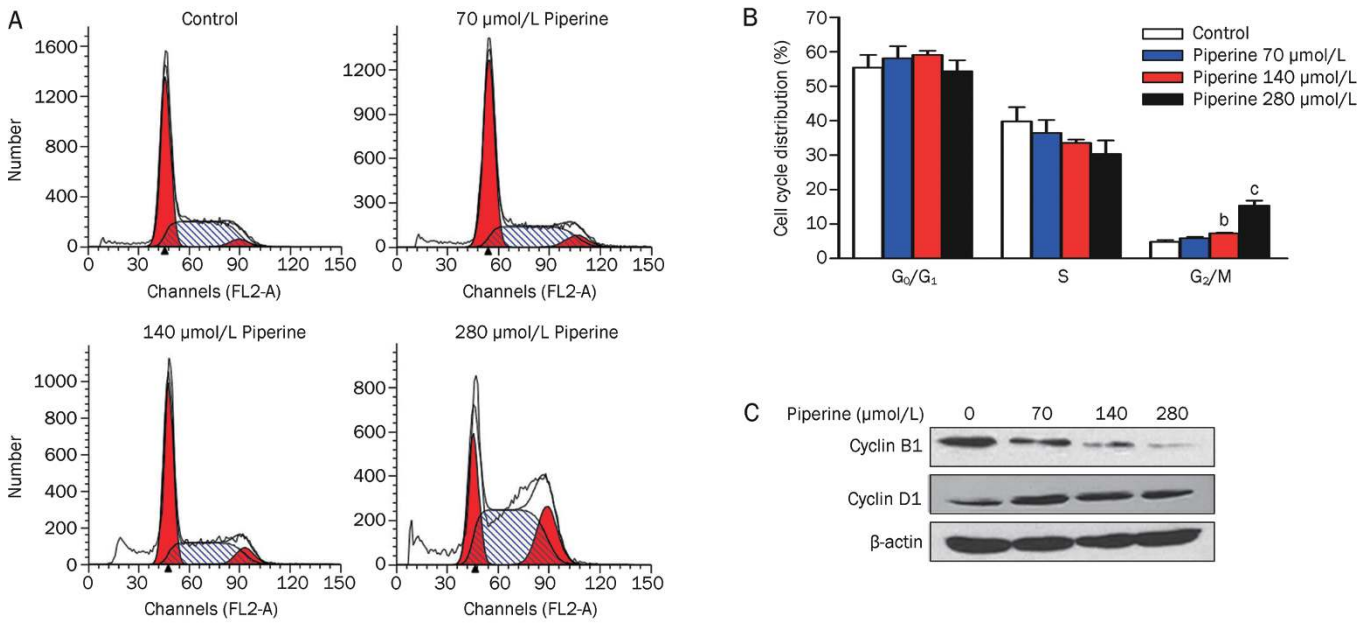

Figure 4. Piperine caused the accumulation of the cells in the G_2/M phase possibly by reducing the expression of cyclin B1. (A) 4T1 cells were treated with piperine 0, 70, 140, 280 μmol/L for 24 h. Then the cells were washed, fixed, stained with propidiumiodide, and analyzed for DNA content by flow cytometry. One representative result was shown. (B) The percentage of cells in different phases of cell cycle after piperine treatment. Mean±SD. n=4. ^bP<0.05, ^cP<0.01. (C) Western blot analysis of cyclin D1 and cyclin B1 expression in 4T1 cells treated with or without piperine. One representative result of three independent experiments was shown.

but also effectively controlled the occurrence of spontaneous metastases.

Discussion

Piperine is a potent anticancer compound that has been

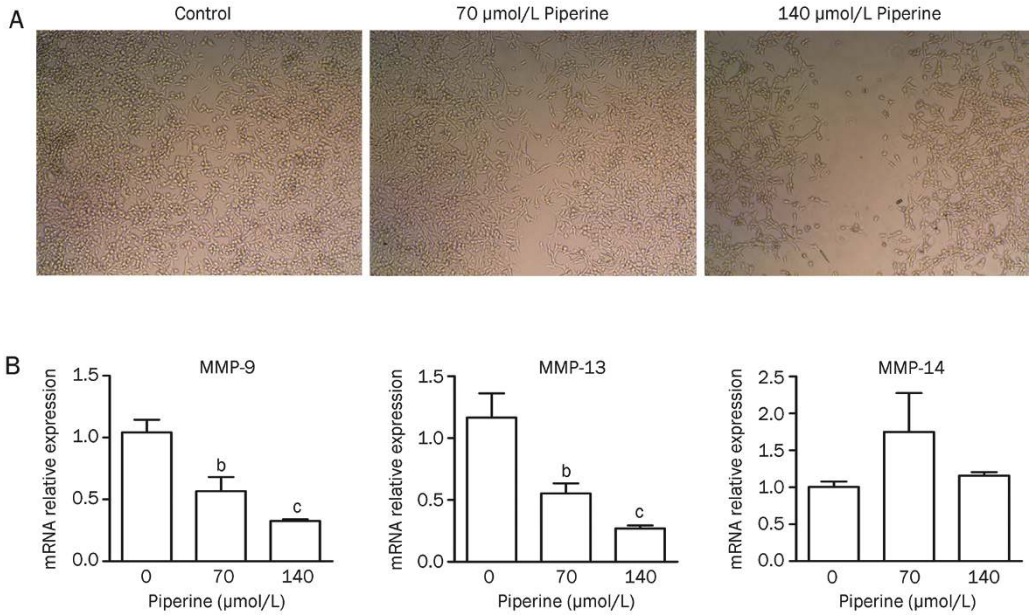

Figure 5. Piperine inhibited 4T1 cell migration *in vitro* and down-regulated the expression of MMP-9 and 13. (A) Cell migration was evaluated by wound healing assay after 24 h incubation with 0, 70, 140 μmol/L piperine. (B) The mRNA expression of MMP-9, 13 and 14 in 4T1 cells treated by piperine at different concentrations (0, 70, and 140 μmol/L) for 24 h was detected by real-time PCR and normalized to the expression of GAPDH in each sample. Mean±SD. n=4. ^{b}P<0.05, ^{c}P<0.01.

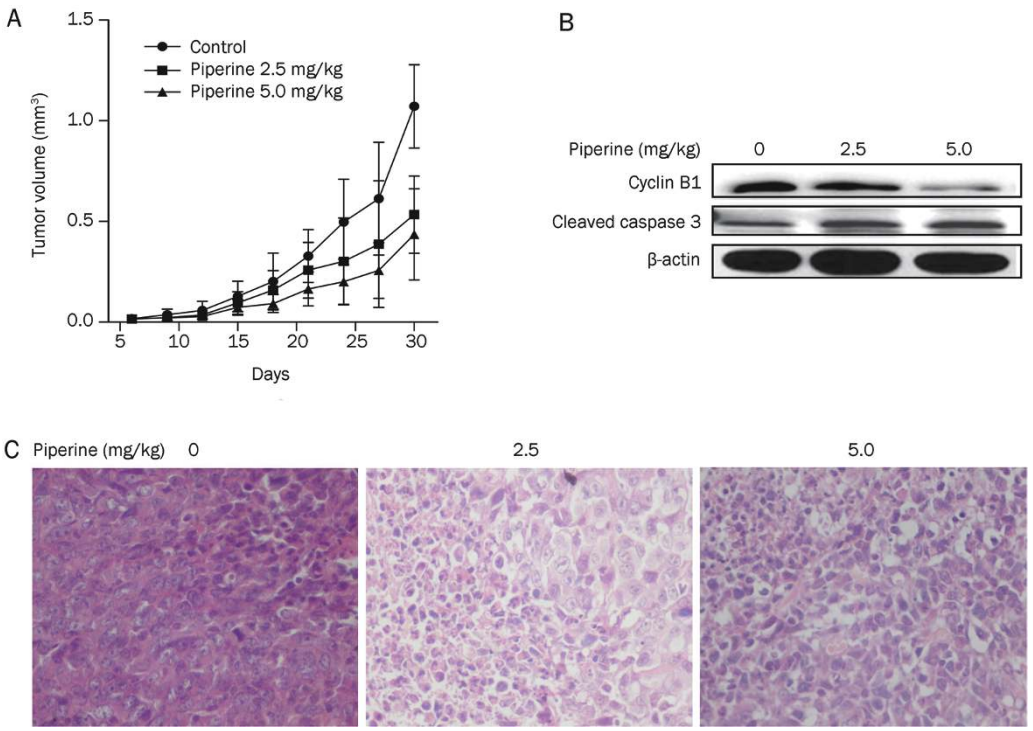

Figure 6. Piperine suppressed 4T1 tumor growth *in vivo*. (A) 5×10^5 cells in 0.1 mL PBS were implanted subcutaneously into the BALB/c mice. After 3 d of implantation, piperine (0, 2.5, and 5 mg/kg) were dissolved in 0.2% DMSO and injected into tumors every three days for three times. The tumor volume was measured every two or three days using the formula $V=0.5236\times d_1^2\times d_2$, where d_1 is the shortest diameter, and d_2 is the longest diameter (n=5). Data are the mean±SD of one representative experiment. Similar results were obtained in at least three independent experiments. (B) Western blot analysis of cleaved caspase 3 and cyclin B1 expression in tumor tissues that were collected on d 20. One representative result of three independent experiments was shown. (C) Hematoxylin-eosin staining of tumor mass after various therapies. Tumor tissues from different groups were collected on d 20, fixed in 10% formalin, and embedded in paraffin. Sections (5 μm thick) were prepared for HE staining.

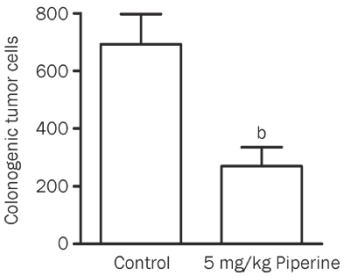

Figure 7. Piperine inhibited the metastasis of 4T1 tumors *in vivo*. 4T1 cells were implanted subcutaneously into the female BALB/c mice. After 3 days of implantation, piperine (0 and 5 mg/kg) was dissolved in 0.2% DMSO and intratumorally injected every three days for three times. Primary tumor was surgically removed on d 15 and mice were sacrificed on d 30 post-tumor inoculation for analysis of lung metastases by colonogenic assay. Mean±SD. n=3. ^bP<0.05.

demonstrated in various cell types. However, the antitumor activity and action mechanism of piperine on mammary cancer cells still remain unknown. Our results firstly indicated that piperine possessed significant cytoxicity against 4T1 cells both *in vitro* and *in vivo*. It was shown piperine induced the apoptosis of 4T1 cells in a dose-dependent manner. We investigated the effect of piperine on the expression levels of the Bcl-2 family, the pro-apoptotic Bax and the anti-apoptotic Bcl-2, which regulate mitochondrial apoptosis. When Bax is overexpressed in cells, apoptotic death in response to death signals is accelerated, while Bcl-2 is overexpressed, it heterodimerized with Bax and the cell death is repressed. Following the exposure to piperine, we found no change of Bax and Bcl-2 at protein level in the 4T1 cells, indicating that the apoptosis induced by piperine is not dependent of the Bcl-2 pathway. We found that the up-regulation of caspase 3 activity may lead to the apoptosis in 4T1 cells. Members of the cyclin family of proteins are key regulators of the cell cycle. Cyclins bind and activate members of the cyclin-dependent kinase (Cdk) family to control cell cycle progression. During the G_1 phase, cyclins D1, D2, and D3 form complexes with cdk4 or cdk6, and cyclin E with cdk2, to modulate the expression of proliferative genes. Cyclin A associates with cdk2 during the S phase, and with cdc2 (cdk1) at the S-G_2 boundary and into G_2. Progression through G_2, culminating in mitosis, further requires that cdc2 form complexes with cyclins B1 and B2[18]. We found that piperine treatment down-regulated the expression of cyclin B1 in 4T1 cells with a dose-dependent manner, while there was no change of cyclin D1 expression after piperine treatment. It is consistent with the observation that the percentage of 4T1 cells in G_2/M phase was increased after piperine treatment, while no significant change in G_0/G_1 phase cell population.

The results of the present study provide the potential application of piperine in tumor therapy. Bhat BG *et al* reported the metabolism of piperine including absorption, tissue distribution and excretion of urinary conjugates in rats[19]. Upon administration of piperine to male albino rats by gavage or intraperitoneally, about 97% was absorbed irrespective of the mode of dosing. Examination of the passage of piperine through the gut indicated that the highest concentration in the stomach and small intestine was attained at about 6 h[19].

The anticancer activity of piperine *in vivo* was probably partly due to its direct antiproliferative effect on 4T1 cells possibly by increasing the caspase 3 activity and down-regulated the expression of cyclin B1. In this study, we also provided the evidence that piperine can suppress the metastasis of 4T1 tumor *in vitro* and *in vivo*. It is well known the process of tumor cell metastasis requires the degradation of ECM molecules in the basement membrane, which is the largest barrier between cancer cells and the bloodstream. The key proteases that are involved in ECM degradation contain MMPs. Our results showed that Piperine significantly reduced the mRNA expression of MMP-9 and MMP-13. It had been reported that suppression of the expression of MMP-9 in tumor cells by piperine is through the inhibition of PKCα and ERK phosphorylation and reduction of NF-κB and AP-1 activation[11]. MMP-13 was shown to be expressed in more invasive breast carcinoma cells[20], and some evidence demonstrated that increased tumor-derived MMP-13 expression independently predicts poor prognoses[21]. In breast cancer, MMP-9 seems to be expressed in cancer tissue. Malignant breast tumors have increased MMP-9 activity compared to the benign tumors[22]. Nevertheless, MMP-9 expression has also been described as a positive prognostic marker in node-negative breast cancer[23]. In another study, positive stromal MMP-9 expression predicts poor survival in the hormone-responsive small tumors, whereas MMP-9 expression in carcinoma cells favors survival[24]. Thus, MMP-9 expression is associated with both inhibition and stimulation of tumor growth and progression. So, the exact mechanism of the inhibitory effect of piperine on tumor metastasis *in vivo* needs to be further elucidated. It have been supposed that the inhibition effects of piperine on oxygenase, p450 isoenzyme and cycloxygenase-1 expression may contribute to the antimetastatic qualities[25].

According to some authors, the antitumor activity of piperine may be related to its immunomodulatory properties, which involves the activation of cellular and humoral immune responses[26, 27]. It was proved to be a potent inhibitor of NF-κB, c-Fos, CREB, ATF-2, and proinflammatory cytokine gene expression in B16F-10 melanoma cells[10]. Other researches documented the chemopreventive efficacy of Piperine is probably due to their antilipidperoxidative and antioxidant potential as well as its modulating effect on the carcinogen detoxification process[28, 29]. Piperine is also known to enhance the bioavailability of some drugs by inhibiting drug metabolism or by increasing absorption[12, 30, 31]. Piperine with other antitumor drugs such as 5-FU[12], piplartine[13] augmented the antitumor activity and also decreased the side effects.

In conclusion, piperine can inhibit tumor growth by inducing cell apoptosis and cell cycle blockage. Also it can suppress 4T1 tumor growth and metastasis *in vivo*. Further studies will need to be carried out to determine the exact mechanisms of the antitumor action of piperine, and to investigate how it can

be used in cancer therapy alone or combination with other antitumor drugs.

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Author contribution

Jian-gen SHEN, Qing-qing WANG, and Li-hua LAI designed research; Li-hua LAI, Qi-hong FU, Yang LIU, Kai JIANG, Qing-ming GUO, Qing-yun CHEN, and Bin YAN performed research; Li-hua LAI and Qi-hong FU analyzed data; Li-hua LAI and Qing-qing WANG wrote the paper.

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