Piperine inhibits proliferation of human osteosarcoma cells via G2/M phase arrest and metastasis by suppressing MMP-2/-9 expression

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ABSTRACT

The piperidine alkaloid piperine, a major ingredient in black pepper, inhibits the growth and metastasis of cancer cells both in vivo and in vitro, although its mechanism of action is unclear. Furthermore, its anticancer activity against osteosarcoma cells has not been reported. In this study, we show that piperine inhibited the growth of HOS and U2OS cells in dose- and time-dependent manners but had a weaker effect on the growth of normal hFOB cells. Piperine inhibited osteosarcoma cell proliferation by causing G2/M phase cell cycle arrest associated with decreased expression of cyclin B1 and increased phosphorylation of Cyclin-dependent kinase-1 (CDK1) and checkpoint kinase 2 (Chk2). In addition, piperine treatment inhibited phosphorylation of Akt and activated phosphorylation of c-Jun N-terminal kinase (c-JNK) and p38 mitogen-activated protein kinase (MAPK) in HOS and U2OS cells. Piperine induced colony formation in these two cell types. We proved that piperine could suppress the metastasis of osteosarcoma cells using scratch migration assays and Transwell chamber tests. Moreover, gelatin zymography showed that piperine inhibited the activity of matrix metalloproteinase (MMP)-2/-9 and increased the expression of tissue inhibitor of metalloproteinase (TIMP)-1/-2 and down-regulation of MMP-2/-9. These findings support further study of piperine as a promising therapeutic agent in the treatment of osteosarcoma.

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1. Introduction

Osteosarcoma is one of the most common types of malignant bone tumors originating from mesenchymal tissue. It accounts for 20% of primary malignant bone tumors and occurs mostly in adolescents and young adults. Osteosarcoma is characterized by metastasis and firm resistance to chemotherapy [1]. However, with the development and use of multi-agent chemotherapy followed by surgical resection, the 5-year survival rate of patients has increased by 60–70% [2]. However, osteosarcoma patients with metastasis have a 5-year survival rate of only 20%, and current therapies have a limited effect in the treatment of recurrent and metastatic osteosarcoma [3]. In 2009, Chen et al. [4] reported that the 5-year survival rate for patients from the time of first pulmonary metastasectomy was up to 36%. Hence, metastasis is still a severe challenge for clinical practitioners in cancer treatment.

The invasion and metastasis of cancer are important biological characteristics of tumor cells [5]. Metastasis is the culmination of tumor progression, and most cancer deaths are due to the progression of metastases [6]. Mortality in osteosarcoma patients results principally from pulmonary metastasis. In metastasis, cancer cells invade other tissues via a chain of complex mechanisms involving several signaling pathways that allow the detachment of primary cancer cells, motility, degradation of the extracellular matrix (ECM), invasion, migration, adhesion to endothelial cells and growth at a new site [7,8]. The vital steps of metastasis require degradation of the ECM by proteolytic enzymes [9]. Matrix metalloproteinases (MMPs), a family of proteinases that can degrade the ECM, including MMP-2/-9, are a group of zinc-dependent endopeptidases involved in angiogenesis and tissue remodeling. They play a significant role in cell migration and invasion by controlling degradation of the ECM [10,11]. MMP-2 is regulated by tissue inhibitor of metalloproteinase (TIMP)-2, and the specific inhibitor of MMP-9 is TIMP-1. Elucidation of MMP-2/-9, TIMP-1 and TIMP-2 activation mechanisms will help us to understand the process of cancer metastasis [12].

The piperidine alkaloid piperine, a major ingredient in black pepper, inhibits the growth and metastasis of cancer cells both in vivo and in vitro. It promotes digestion by stimulating gastric acid secretion and increasing pancreatic and intestinal lipase activities [13,14]. In addition, some studies reported that piperine has anti-inflammatory [15,16],
neuroprotective [17], antioxidant [18] and cardiovascular protective effects [19]. Paul reported that piperine inhibits colon cancer cell growth and causes apoptosis by triggering endoplasmic reticulum stress [20]. In addition, piperine has been reported to lower cancer incidence in animal models of lung cancer [21,22]. When combined with curcumin, piperine significantly increased the cytotoxicity of curcumin in breast stem cells [23]. Recent studies suggest that piperine inhibits angiogenesis by inhibiting the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in endothelial cells [24]. Signaling pathways involved in regulating cancer cell invasion and MMP expression include mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways [7,8]. The above studies suggest that piperine may be a promising agent for cancer treatment.

Despite its use and ability to inhibit several cancer types, no findings on the effects of piperine against osteosarcoma have been reported. Therefore, in this study, we focused on the in vitro anti-proliferative and anti-metastatic effects of piperine on human osteosarcoma HOS and U2OS cells and the possible molecular mechanisms involved. Our results show that piperine causes G2/M cell cycle arrest and inhibits MMP-2/-9 expression and activation through MAPK and Akt signaling pathways, thereby suppressing HOS and U2OS cell proliferation, migration and invasion. Our findings provide a potential mechanism and an experimental foundation for the clinical treatment of osteosarcoma using piperine.

2. Materials and methods

2.1. Cell culture

HOS, hFOB and U2OS cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and maintained at 37 °C in a 5% CO2 atmosphere in DMEM and RPMI 1640 medium, respectively. The medium was supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 mg/mL streptomycin.

2.2. Reagents and antibodies

Piperine (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mM and stored at −20 °C. The molecular formula of piperine is C17H19NO3, and its molecular weight is 285.34 (Fig. 1A). Primary antibodies against p-Akt (Ser473), p-JNK (Thr183/Tyr185), p-ERK (Thr202/Tyr204), cyclin B1, p-CDK1 (Tyr15), p-Chk2 (Thr68), TIMP-1, TIMP-2 and GAPDH were purchased from Cell Signaling Technology (Beverly, MA). Matrigel was obtained from BD Biosciences (NJ, USA), Wortmannin (PI3K/Akt inhibitor), SB202190 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) were purchased from Selleck Chemicals (Houston, TX).

2.3. Cell viability assay

HOS and U2OS cells were seeded in 96-well flat-bottomed tissue culture plates (4,000 cells/well) and incubated at 37 °C in 5% CO2 with different concentrations of piperine (25–400 μM) for 24, 48 and 72 h. Cell viability was determined using MTS kit (cellTiter96AQ, Promega, USA). The OD value was measured at 490 nm using an MR7000 microplate reader (Dynatech).

2.4. Cell cycle analysis

HOS and U2OS cells were plated at 2.5 × 104/well in 6-well plates and treated with piperine. After 72 h, the cells were then harvested with trypsin, washed, resuspended in cold PBS and fixed in cold 75% ethanol for storage at −20 °C overnight. Next, the cells were washed and resuspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase (BD Biosciences, NJ, USA) and then incubated for 15 min at room temperature. PI-stained cells were analyzed using flow cytometer and ModFit LT software (FACS-Calibur).

2.5. Colony formation assay

HOS and U2OS cells were plated in 6-well plates at 800/well and 500/well, respectively. After 12 h of culture, the cells were treated with medium alone, vehicle (DMSO) or different concentrations of piperine. After 14 days of culture, the medium was removed, and the cells were washed with PBS before the addition of methanol for 15 min. The cells were then washed with PBS, and 0.1% crystal violet was added for 10 min. Finally, the plates were washed and cell colonies were counted by visual inspection.

2.6. Wound healing assay

The scratch migration assay is a standard method for evaluating drug effects on cell migration in vitro. HOS and U2OS cells were seeded in 6-well plates at a density of 3.5 × 104 and 4.0 × 104 cells/well, respectively, and cultured for 24 h to allow them to reach ~90% confluence. The cells were then scraped carefully using a sterile 100-μl plastic pipette tip to make a scratch. Debris was removed from the cultures, and the cells were treated with piperine. The width of the denuded area was assessed at 0 and 24 h using a phase contrast microscope. The migration rate was calculated using the following equation: migration rate = (average original width − average final width) / average original width × 100%.

2.7. Matrigel invasion assay

HOS and U2OS cells were starved by culturing in serum-free medium for 12 h and then plated in 6-well plates at a density of 2.5 × 105 cells/well. After treatment with piperine for 24 h, the cells were harvested, and their invasiveness was determined using a Transwell chamber (Corning, USA). Matrigel (50 mg/mL) was diluted with serum-free medium at a ratio of 1:8, and each Transwell chamber was coated with 60 μl diluted Matrigel. Before use, the polycarbonate membrane (pore size 8 μm) was hydrated with 50 μl serum-free medium containing 10 g/l BSA at 37 °C for 30 min. Treated cells (8 × 104/well) were then plated into the upper chamber in 200 μl serum-free medium, and 600 μl medium containing 10% FBS was added to the lower chamber as a control. After incubation for 24 h at 37 °C in 5% CO2, the cells that had invaded the lower surface of the filter were fixed with 75% ethanol and stained with 0.1% crystal violet. Cell numbers were counted in three random areas (×400) per chamber.

2.8. Analysis of MMP-2/-9 activities using gelatin zymography

HOS and U2OS cells were treated with different concentrations of piperine for 24 h, and the supernatants were collected for gelatin zymography. The protein concentrations of the supernatants were assayed using BCA assay reagent (Beyotime Institute Biotechnology, China). Samples were mixed with 5 × loading buffer, and equivalent amounts of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 80 V and 4 °C for 3 h. The gels contained 0.1% gelatin as a substrate. After electrophoresis, the gels were removed, rinsed three times and incubated at 37 °C for 48 h in activation buffer (50 mM Tris–HCl, 5 mM CaCl2, 1 mM ZnCl2, 0.02% NaN3). The gels were then stained with 0.05% Coomassie blue R-250 for 3 h and destained using destaining solution until clear bands were visible.

2.9. Western blot analysis

After treatment with piperine, cells were harvested, washed, resuspended in ice-cold RIPA lysis buffer containing 2 mM
phenylmethanesulfonyl fluoride (PMSF) and incubated on ice for 30 min. The suspension was then centrifuged at 13,000 g for 15 min at 4 °C. Protein levels were quantified using BCA assay reagent (Beyotime Institute Biotechnology). Equivalent amounts of protein were resolved by 10% SDS–PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked for 1 h at room temperature using TBST containing 5% w/v BSA and incubated with primary antibodies overnight at 4 °C. After washing, the membranes were incubated with secondary antibody for 1 h at room temperature. Protein bands were detected using the ChemiDoc imaging system (Bio-Rad, USA).

2.10. Statistical analysis

Data are expressed as the means ± SD from experiments performed in at least triplicate. One-way ANOVA was used for statistical analysis. All statistical analyses were performed using SPSS software (ver.16.0; SPSS, Inc., Chicago, IL). P values were two-tailed, and a value <0.05 was considered statistically significant.

3. Results

3.1. Piperine reduces the viability of osteosarcoma cells in time- and dose-dependent manners

To study the effects of piperine on the growth of osteosarcoma cells, we first cultured HOS and U2OS cells with different concentrations of piperine (25–200 μM) for 24, 48 or 72 h. After 72 h, the IC₅₀ values for piperine based on the MTS assay were 72 μM in HOS cells and 126 μM in U2OS cells (Fig. 1B). According to MTS assay, piperine inhibited cell viability in time- and dose-dependent manners (Fig. 1C, D). Meanwhile,
we treated normal human hFOB osteoblasts with the same piperine dose. in 72 h (Fig. 1E). Piperine had a weaker inhibitory effect on the proliferation of hFOB cells compared with osteosarcoma cells. However, we founded that treated by piperine 200 μM in 72 h, the ratio of apoptosis from 6.8% to 22.3% in HOS cells and from 4.4% to 15.5% in U2OS cells, respectively (Fig. 1F). Thus, piperine should be considered relatively safe when applied clinically.

3.2. Piperine causes G2/M phase arrest

Antiproliferative effects can be caused by cell cycle arrest and/or cytotoxicity. We used flow cytometry to analyze the growth inhibition of HOS and U2OS cells cultured for 72 h with different concentrations of piperine. Exposure to piperine resulted in remarkable increases in the percentages of HOS and U2OS cells in the G2/M

Fig. 2. Piperine induces G2/M cell cycle arrest and alters the expression of G2/M phase–related proteins in osteosarcoma cells. (A and C) Representative cell cycle profiles of HOS and U2OS cells treated with piperine for 72 h, (B and D) Populations of HOS and U2OS cells in each cell cycle phase, (E and G) Piperine altered the levels of cyclin B1, p-CDK1 and p-Chk2 in HOS and U2OS cells in dose- and time-dependent manners. (F and H) Relative expression was calculated by densitometric analysis. GAPDH was used as an internal control. The results suggest that treatment with piperine at different concentrations arrested cells in the G2/M phase. Data are expressed as the means ± SD of three independent experiments. *p < 0.05, #p < 0.05, **p < 0.01 and ##p < 0.01 compared with the control group.
phase of the cell cycle. The percentage of cells in the G2 phase increased from 13.3% to 34.86% in HOS cells and from 9.61% to 27.67% in U2OS cells (Fig. 2A–D). Accordingly, the percentages of cells in the G0/G1 phase decreased.

3.3. Piperine inhibits colony formation in osteosarcoma cells

To determine the ability of piperine to inhibit the formation of colonies from single cells, we exposed HOS and U2OS cells to three

![Image of colony formation experiment](image-url)
different doses of piperine for 14 days and performed a colony formation assay (Fig. 3). As the images show, the formation of colonies was inhibited progressively with increasing concentrations of piperine. This suggests that piperine inhibits the clonogenicity of osteosarcoma cells.

3.4. Inhibitory effects of piperine on migration and invasion

We investigated the effects of piperine on the migration and invasion of HOS and U2OS cells using wound healing and Transwell assays. Incubation with different doses of piperine suppressed the migration and invasion of these cells.
of HOS and U2OS cells into the denuded zone (Fig. 4A). The migration rate decreased from 63.6% to 28% in HOS cells and from 73% to 38.5% in U2OS cells (Fig. 4B). The Transwell assay reflects the invasion ability of cells. To illustrate the inhibitory effect of piperine on the ability of HOS and U2OS cells to penetrate the extracellular matrix, we assessed the number of cells that crossed the Matrigel in the Boyden chamber. Piperine suppressed invasion of HOS and U2OS cells through the Matrigel in a dose-dependent manner (Fig. 4C). After treatment with 100 and 200 μM piperine for 24 h, the number of invading cells was reduced from 88 to 20 per field for HOS cells and from 150 to 23 per field for U2OS cells (Fig. 4D). These results indicate that piperine markedly inhibited not only the migration but also the invasion of HOS and U2OS cells.

3.5. Piperine inhibits the expression and activation of MMP-2/-9 in HOS and U2OS cells

Because the expression and activation of MMPs are vital to ECM degradation, which contributes to cell invasion, the effects of piperine on MMP expression and activation were evaluated. Gelatin zymography showed that the enzymatic activities of MMP-2/-9 were decreased significantly after treatment with 100 μM piperine (Fig. 5A). In addition, western blot analysis revealed that treatment with piperine for 24 h reduced the expression of MMP-2/-9 (Fig. 5B). Meanwhile, TIMP-1/-2 expression was increased and CD147 expression decreased (Fig. 5B, C). Taken together, these data suggest that the suppression of MMP-2/-9 expression and activities might contribute to the inhibition of migration and invasion of HOS and U2OS cells by piperine.

3.6. Piperine suppresses proliferation and invasion in HOS and U2OS cells via cyclin B1/CDK1, Akt and MAPK signaling pathways

To investigate the mechanism of G2/M cell cycle arrest, we assessed the G2/M phase-related proteins cyclin B1, CDK1 and Chk2 by Western blotting. Piperine increased the phosphorylation of CDK1 and Chk2 and down-regulated cyclin B1 expression in time- and dose-dependent manners in both HOS and U2OS cells (Fig. 2E–H). These results are in accordance with those of flow cytometry analysis.

MAPK members are vital regulators of pathways related to cell proliferation and migration [25]. Therefore, we evaluated the effects of piperine on p38 and JNK activation and found that piperine increased the phosphorylation of p38 and JNK in a dose-dependent manner in HOS and U2OS cells (Fig. 6). Akt plays an important role in the growth, survival and motility of cancer cells. We assessed the expression of Akt and observed reduced phosphorylation of Akt in piperine-treated HOS and U2OS cells. To investigate the relevance of Akt, p38 and JNK to MMP-2/-9 expression, we applied the specific inhibitor of Wortmannin (P38/Act inhibitor), SB202190 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor). The results showed that Wortmannin with piperine could inhibit the expression of p-Akt obviously but nearly have no difference to expression of MMP-2/-9 compared to piperine group in HOS and U2OS cells after treatment in 48 h. In the experiment with SB202190, piperine with SB202190 group, not only the expression of p-p38 was decreased significantly but also MMP-2/-9 expressions were raised markedly compared to piperine group in HOS and U2OS cells. Meanwhile, we found the similar result to the experiment with SP600125. Therefore, we infer that piperine might prevent HOS and U2OS cell migration and invasion mainly by activating p38 and JNK signaling pathways, thereby reducing MMP-2/-9 expression.

4. Discussion

The phytochemical piperine has been revealed recently to possess anticancer properties, such as anti-proliferative and anti-angiogenic effects. It inhibits invasion and induces apoptosis in a number of cancer cell lines [24,26–28], but its effects on human osteosarcoma cells and its mechanism of action have not been evaluated. In this study, we showed that piperine inhibited the growth of osteosarcoma cells via G2/M phase cell cycle arrest and inhibited their invasion by reducing expression of MMP-2/-9. These effects are associated with MAPK and Akt signaling pathways.

In eukaryotic cells, cell cycle checkpoints play a key role in regulating cell cycle transition, and abnormal regulation of cell cycle checkpoints often occurs in cancer cells. After DNA damage, cell cycle arrest is regulated by cell cycle checkpoints. When cellular damage is irreversible, apoptotic pathways might be activated, leading to cell death. As two apical checkpoint kinases involved in the response to DNA damage, ATM and ATR directly phosphorylate the checkpoint transducer kinases CHK2 and CHK1, respectively. ATM-CHK2 activates the p53-p21 pathway, p21 being an inhibitor of cyclins/CDKs that regulates cell cycle arrest [29]. Thus, when cells undergo G2/M phase arrest, the expression of cyclin B1/CDK1 is reduced [30].

Our study shows that in osteosarcoma cells treated with piperine for 72 h, the G1 phase cell population decreased with little change in the S phase population. The G2 phase cell population was increased significantly compared with the control group. These data indicate that piperine induced cell cycle arrest by inducing G2/M phase arrest, rather than G1 or S phase arrest. Western blot analysis showed that piperine reduced the expression of cyclin B1 in dose- and time-dependent manners. Likewise, levels of the related proteins p-CDK1 and p-Chk2 were altered in HOS and U2OS cells treated with piperine. By contrast, Chien recently reported that G2/M arrest in colorectal carcinoma cells treated with evodiamine for 24 h was associated with increased expression of cyclin B1 [31]. Through further analysis, we found that cyclin B1 may have undergone corresponding changes at different time points. Therefore, our data are not in conflict with those of others. We found a remarkable effect of piperine on cell cycle distribution in osteosarcoma cells.

MMPs belong to a group of zinc ion-dependent endopeptidases that are related to the degradation of ECM proteins in tumorigenic processes and that facilitate cancer cell migration and invasion. In addition, they coordinate the host defense system and normal cell functioning [32]. Among them, MMP-2/-9 play a vital role in tumor cell migration and invasion. CD147, also named extracellular matrix metalloproteinase inductive (EMMPRIN), is a highly glycosylated transmembrane protein with two Ig domains that belongs to the immunoglobulin superfamily [33]. Previous studies clearly indicate that CD147 is highly expressed in tumor cells and stimulates MMP secretion and activation in stromal cells and/or tumor cells, thereby resulting in ECM degradation and promoting tumor progression and metastasis [34]. Zhou found that MMP-2/-9 were induced by CD147 and promoted the invasive ability of THP-1 cells; conversely, CD147 inhibition reduced the invasive ability of THP-1 cells by reducing the expression of MMP-2/-9 [33]. In addition, siRNA-mediated silencing of CD147 in lymphoma cells inhibited tumor progression and improved sensitivity to chemotherapy [35]. Our results indicate that 50–200 μM piperine inhibited the activities and expression of MMP-2/-9 after 24 h. It also decreased CD147 expression and significantly up-regulated TIMP-1/-2 expression. These results demonstrate that the suppression of CD147 and MMP-2/-9 expression and enzymatic activities might be responsible for the anti-metastatic effect of piperine.

Fig. 6. Piperine up-regulates the phosphorylation of JNK and p38 and down-regulates the phosphorylation of Akt in human osteosarcoma cells and reduces MMP-2/-9 expression mainly via JNK and p38 pathways. (A) Levels of p-JNK, p-p38 and p-Akt in HOS and U2OS cells after treatment with piperine for 48 h. (C, E and G) Addition of Wortmannin, SB202190 and SP600125 could decrease the expression of p-Akt, p-p38 and p-JNK (E and G) and recovery of MMP-2/-9 expression. (B, D, F and H) Relative expression was calculated by densitometric analysis. GAPDH was used as an internal control. Data are expressed as the means ± SD of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.01 compared with the control group.
A number of reports suggest that MMP-2 and MMP-9 expression is relevant to the MAPK family and the Akt/mTOR pathway [36–38]. Moreover, the inhibition of PI3K/Akt and NF-κB pathways suppressed MMP expression [8,39]. A recent study found that the activation of three major MAPK pathways and the inhibition of the Akt/mTOR pathway reduced MMP-2/-9 expression and prevented prostate cell migration [40]. To elucidate the signaling pathways underlying piperine-mediated responses in osteosarcoma cells, we further investigated the effects of piperine on the activation of MAPK and Akt pathways. We found that piperine inhibited the phosphorylation of Akt and activated the phosphorylation of p38 and JNK in dose-dependent manners in both HOS and U2OS cells. From the further study revealed that Wortmannin inhibited the Akt expression, but have not been a correlative change to MMP-2/-9 expression; however, the inhibition of p38 and JNK expression have a negative correlation to MMP-2/-9 expression. Hence, we speculate that piperine reduces MMP-2/-9 expression mainly by activating MAPK pathways, thereby suppressing the metastasis of osteosarcoma cells.

In conclusion, our study found that piperine inhibits migration and invasion by suppressing the activities and expression of MMP-2/-9. These effects are related to MAPK pathway activation and Akt pathway inhibition. Our results suggest that piperine may be a promising agent for osteosarcoma patients. However, further studies are needed to verify the in vivo effects of piperine on osteosarcoma.

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