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# Inhibition of osteosarcoma cell proliferation by matrine (MT) via targeting histone H3 lysine 27 trimethylation and AMPK activation

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*Received August 16, 2020, accepted September 18, 2020*

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*Pharmazie 76: 38-41 (2021)* *doi: 10.1691/ph.2021.0718*

In the current study effect and mechanism of matrine (MT) on osteosarcoma cell growth was investigated. Matrine treatment led to a significant suppression of U2-OS and MG-63 cell proliferation in the 0.5–15 μM concentration range. In matrine (15 μM) treated U2-OS and MG-63 cells, apoptosis increased to 58.92 and 61.76%, respectively at 48 h. Cleaved caspases-3 as well as Bax levels were markedly elevated in matrine treated U2-OS and MG-63 cells. Besides, Bcl-2 was suppressed in U2-OS and MG-63 cells under treatment with matrine. Treatment with matrine caused a marked elevation in phosphorylation of AMPKα in U2-OS and MG-63 cells. Additionally, H3K27me3 expression as well as EED, EZH2 and SUZ12 levels were inhibited in U2-OS and MG-63 cells by matrine treatment. In summary, matrine inhibits osteosarcoma cell viability by activation of apoptosis. Moreover, matrine activates AMPK and downregulates Histone H3 lysine 27 trimethylation in osteosarcoma cells. Therefore, matrine can be used as therapeutic candidate for osteosarcoma treatment.

## 1. Introduction

Osteosarcoma is the malignant tumor of bones prevalent in adolescents and children (Xing et al. 2019, Wang et al. 2019). There has been great advancement in the treatment of osteosarcoma but metastasis and tumor recurrence continue to remain uncontrolled. Tumor recurrence after surgery and metastasis to pulmonary tissues contribute towards the poor prognosis of osteosarcoma and average 5-year survival of patients with metastatic stage is below 20%. For the treatment of malignant tumors of bones comprehensive strategies consisting of surgery supplemented with radio- and chemotherapies are used (Zohreh et al. 2019). However, application of these treatments leads to harmful effects in the body and not all patients can afford this type of treatment. Thus, the development of novel drugs with anti-tumor potential and negligible or no toxicity is required.

Mitochondria are important cell organelles, which, besides regulating energy metabolism, are also playing a vital role in apoptosis of cells (Chan 2006). Anticancer agents generally target functioning of mitochondria as well as its downstream biogenetics to arrest tumor progression (Neuzil et al. 2013). The number of mitochondria is significantly reduced in many cancer cells like gastric, renal and breast carcinoma cells (Yu et al. 2007; Xing et al. 2008). Osteosarcoma cells are also containing a lower number of mitochondria than musculoskeletal and normal muscular cells (Onishi et al. 2014).

Plant derived secondary metabolites exhibit a protective role against different diseases including inflammatory disorders (Jeyaseelan et al. 2005; Kazemi et al. 2018). Many compounds obtained from the extract of the medicinal herb *Sophora flavescens* Ait were also found to possess good pharmacological activities (Blanco et al. 2005). Oxtymatrine isolated from this herb has shown potential anti-inflammatory activity and is being used for the treatment of various diseases (Blanco et al. 2005). Other compounds such as matrine have shown hepato-protective, oxidant radical quenching and anti-inflammatory activities. Additionally, some compounds from this plant are preventing liver inflammation, brain injury induced by trauma and acute pancreatitis (Yu-Ping et al. 2011; Yao et al. 2005). The current study investigated matrine (Fig. 1) for the treatment of osteosarcoma *in vivo* and also evaluated potential underlying mechanisms.

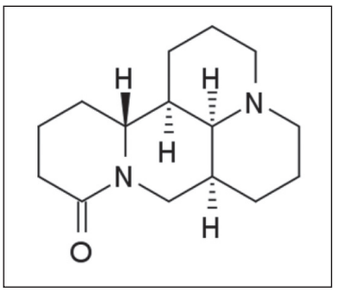


Fig. 1: Chemical structure of matrine (MT).

## 2. Investigations and results

### *2.1. Inhibition of U2-OS and MG-63 cell proliferation by matrine*

Proliferation changes by matrine treatment at 0.5, 1.5, 3, 6, 12 and 15 μM doses in U2-OS and MG-63 osteosarcoma cells and hOB normal osteoblasts were determined by MTT assay (Fig. 2). Matrine treatment of U2-OS and MG-63 cells reduced viability in dose based manner at 48 h compared to control. However, hOB cell viability was not affected on treatment with 0.5, 1.5, 3, 6, 12 and 15 μM doses of matrine. Treatment with matrine at 0.5 and 15 μM doses reduced U2-OS cell viability to 89 and 32%, respectively. The viabilities of MG-63 cells decreased to 84 and 27%, respectively, on treatment with 0.5 and 15 μM doses of matrine.

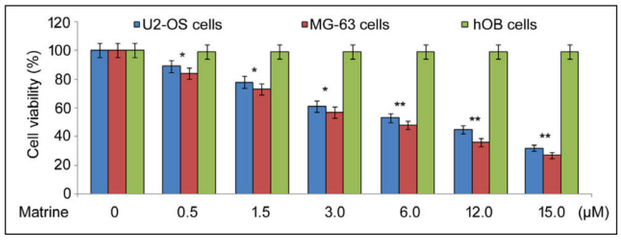


Fig. 2: Matrine suppresses U2-OS and MG-63 cell proliferation. Osteosarcoma cells (U2-OS and MG-63) and normal osteoblasts (hOB) were treated with 0.5, 1.5, 3, 6, 12 and 15 μM doses of matrine for 48 h. The viabilities of cells were assayed by MTT assay. *p<0.05 and **p<0.02 vs. control cells.

### 2.2. Increase in U2-OS and MG-63 cell apoptosis by matrine

Apoptotic effect of 12 and 15 μM doses of matrine on U2-OS and MG-63 cells at 48 h were detected by flow cytometry (Fig. 3). Treatment with matrine significantly ($p < 0.5$) enhanced apoptosis in osteosarcoma cells compared to control cells. Apoptosis in U2-OS cells increased to 43.67 and 58.92%, respectively, on treatment with matrine at 12 and 15 μM doses. In MG-63 cells treatment with 12 and 15 μM doses of matrine raised apoptosis to 47.21 and 61.76%, respectively.

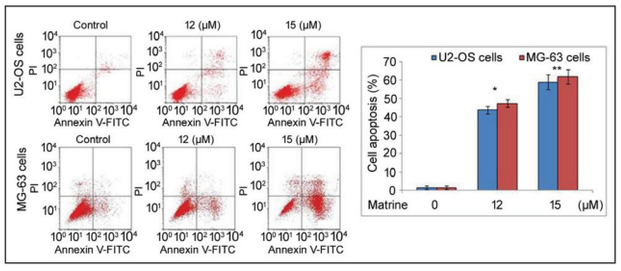


Fig. 3: Effect of matrine on U2-OS and MG-63 cell apoptosis. (A) The cells were treated for 48 h with 12 and 15 μM doses of matrine and apoptosis was assayed by flow cytometry. (B) Data from flow cytometry was quantified. *p<0.05 and **p<0.02 vs. control cells.

### 2.3. Matrine elevates pro-apoptotic proteins in U2-OS and MG-63 cells

Treatment of U2-OS and MG-63 cells with matrine at 12 and 15 μM doses was followed by evaluation of caspases-3, Bax and Bcl-2 proteins by western blotting (Fig. 4). Treatment with matrine markedly elevated expression of cleaved caspases-3 and Bax in U2-OS and MG-63 cells relative to control cells. Moreover, matrine treatment inhibited Bcl-2 expression in U2-OS and MG-63 cells.

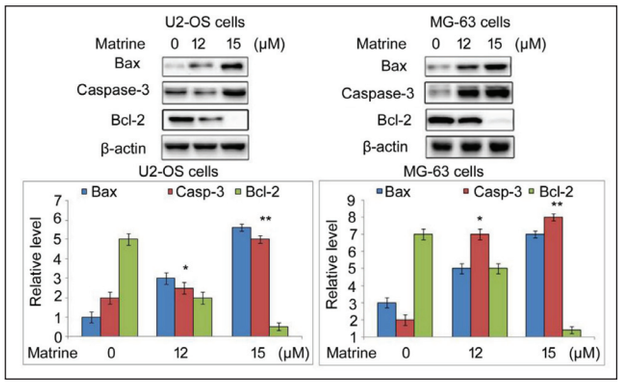


Fig. 4: Effect of matrine on apoptotic proteins in U2-OS and MG-63 cells. The cells were treated with matrine at 12 and 15 μM doses for 48 h and expression of proteins was assayed by western blotting. The protein expression was quantified. *p<0.05 and **p<0.02 vs. control cells.

### 2.4. Matrine promotes p-AMPK expression in U2-OS and MG-63 cells

Phosphorylation of AMPKα by matrine treatment in osteosarcoma cells was evaluated by MTT assay (Fig. 5). Treatment with 12 and 15 μM doses of matrine caused a marked elevation in phosphorylation of AMPKα in U2-OS and MG-63 cells compared to control cells.

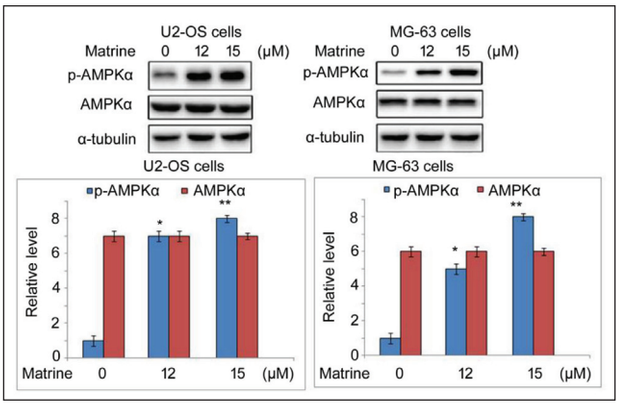


Fig. 5: Effect of matrine on phosphorylation of AMPKα in U2-OS and MG-63 cells. The cells were treated for 48 h with 12 and 15 μM doses of matrine and phosphorylation of AMPKα was evaluated by western blotting. The protein expression was quantified. *p<0.05 and **p<0.02 vs. control cells.

### 2.5. Inhibition of H3K27me3 by matrine in U2-OS and MG-63 cells

The expression of H3K27me3 in U2-OS and MG-63 cells at 48 h of treatment with matrine was evaluated by western blotting (Fig. 6). Treatment with 12 and 15 μM doses of matrine significantly reduced H3K27me3 expression in osteosarcoma cells. Moreover, matrine treatment also reduced the levels of EED, EZH2 and SUZ12 expression in U2-OS and MG-63 cells.

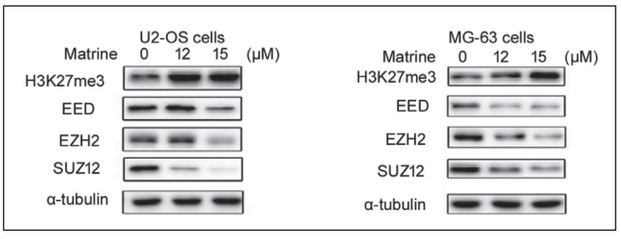


Fig. 6: Effect of matrine on H3K27me3 expression in U2-OS and MG-63 cells. (A) Cells after treatment with 12 and 15 μM doses of matrine for 48 h were assayed for expression of indicated proteins by Western blotting. (B) Proteins were quantified. *p<0.05 and **p<0.02 vs. control cells.

## 3. Discussion

Osteosarcoma is the primary bone cancer common in children and adolescents with very high metastatic rate. Treatment strategies for osteosarcoma consist of surgery and combination of chemotherapeutic agents (Friebele et al. 2015; Mirabello et al. 2009). The major limitation to presently available treatments is the high rate of metastasis of the osteosarcoma cells. Patients suffering from pulmonary metastatic osteosarcoma have less than 20% average 5-year survival rate because of resistance to therapies and very high metastatic potential (Zhang et al. 2019). Thus, novel drugs and more effective treatments are for osteosarcoma are urgently required.

High rate of osteosarcoma proliferation and metastasis comprising local adhesion, cell migration, invasion is linked to poor prognosis (Hsu et al. 2019; Nourmohammadi et al. 2019). In the current study, matrine treatment suppressed proliferation of U2-OS and MG-63 cells significantly in dose dependent manner. However, viability

of normal osteoblasts (hOB) was not affected on treatment with various doses of matrine. Moreover, matrine treatment upregulated apoptosis in U2-OS and MG-63 cells significantly compared to control. Matrine treatment markedly elevated expression of cleaved caspases-3 and Bax in U2-OS and MG-63 cells relative to control cells. Moreover, in matrine treated U2-OS and MG-63 cells Bcl-2 expression was inhibited and p21 level increased compared to control.

The enzyme AMPK which regulates energy utilization is associated with cancer cell growth through its interaction with various cell cycle checkpoints (Sanli et al. 2010). Basically, AMPK maintains energy stores in the cell by controlling oxidative metabolism, inhibition of consumption of ATP and regulation of mitochondria biogenesis (Jäger et al. 2007). Direct upregulation of PGC-1α expression by AMPK plays a vital role in regulating the process of mitochondrial biogenesis (Jäger et al. 2007). Studies have demonstrated that AMPK promotes FOXO3a activation and increases p53 expression leading cell apoptosis or cell cycle arrest (Gwinn et al. 2008; Shackelford et al. 2009). Increased levels of cyclin-dependent kinase inhibitors such as p21cip1 and p27kip1 by activated AMPK is associated with arrest of cell cycle and its anti-tumor effect (Gwinn et al. 2008; Mihaylova and Shaw 2011). In the present study, matrine treatment of U2-OS and MG-63 cells markedly elevated the phosphorylation of AMPKα compared to control cells. Increase in phosphorylation of AMPKα in U2-OS and MG-63 cells on treatment with matrine suggested its osteosarcoma inhibitory potential.

Downregulation of enhancer of zeste (EZH2) transcription by antitumor agents has been found to inhibit the pancreatic and breast cancer growth *via* promotion of miR-26a and miR-101 expression (Bao et al. 2012; Cabello et al. 2016). The present study revealed that matrine treatment of U2-OS and MG-63 cells targeted EZH2, SUZ12 and EED expression. In matrine treated osteosarcoma cells, a significant reduction was observed in expression of EZH2, SUZ12 and EED proteins. Inhibition of histone H3 methylation at Lys9 in prostate cancer cells by metformin leads to a significant suppression of migration potential (Yu et al. 2017). In the current study, expression of tri-methylated histone protein (H3K27me3) was evaluated in matrine treated U2-OS and MG-63 cells. Treatment of U2-OS and MG-63 cells with matrine markedly down regulated the expression of H3K27me3 relative to control cells. Thus present study demonstrated that matrine inhibits U2-OS and MG-63 cell proliferation and activated apoptosis but did not affect normal osteoblast (hOB) viability. Moreover, matrine treatment of osteosarcoma cells elevated phosphorylation of AMPKα and targeted enhancer of zeste expression. Therefore, matrine could be developed as therapeutic agent for the treatment of osteosarcoma.

## 4. Experimental

### *4.1. Cell lines*

Osteosarcoma cell lines (U2-OS and MG-63) and normal osteoblasts (hOB) were supplied by the Cell Bank belonging to Chinese Academy of Sciences (Shanghai, China). The cell cultures were performed for 24 h in DME-medium containing 10% FBS and 1% penicillin-streptomycin. Cell incubation was carried out with 0.5, 1.5, 3, 6, 12 and 15 µM doses of matrine for 48 h under a humidified atmosphere containing 5% $CO_2$ at 37 °C.

### *4.2. MTT assay*

The U2-OS, MG-63 and hOB cells were distributed into 96-well plates in DMEM containing 10% FBS supplemented with 1% penicillin-streptomycin at 4 x $10^5$ cells/ well density. After culture for overnight at 37 °C the medium in wells was changed by fresh medium containing 0.5, 1.5, 3, 6, 12 and 15 µM doses of matrine. After 48 h, 20 µl volume of MTT (5 mg/ml) solution was added to the wells and incubation was performed for 4 h more at 37 °C. Cell viability measurements were made after formazan crystals were dissolved by adding dimethyl sulfoxide solvent. Absorbance of the wells was determined at 567 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### *4.3. Flow cytometry*

The U2-OS and MG-63 cells were treated with 12 and 15 µM doses of matrine for 48 h at 2 x $10^5$ cells/ well density in 6-well plates. The cells washed with PBS were fixed with 1% paraformaldehyde plus PbS and subsequently suspended in ethyl alcohol (70%). Then Annexin V-FITC (5 µl) and PI (5 µl) were added to the cells and incubation was performed in the dark for 5 min at 37 °C. A flow cytometer BD Accuri C6 Plus (BD Accuri™; BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze apoptosis of the cells.

### *4.4. Western blotting analysis*

The U2-OS and MG-63 cells were treated with 12 and 15 µM doses of matrine for 48 h, washed with PBS and then lysed on treatment with RIPA buffer containing protease inhibitors. The lysate was centrifuged at 4 °C for 15 min at 16,000 x g and protein concentration in the supernatants was determined by Bradford Protein Assay kit. The protein samples (30 µg) were subjected to resolution on 10% SDS-PAGE by electrophoresis and subsequently transferred to a PVDF membrane using Trans-Blot®SD Semi-Dry Transfer Cell. Blocking of non-specific sites in membranes was performed by incubation at 4 °C with 5% skimmed milk powder for overnight. Then incubation was carried out for overnight at 4 °C with primary antibodies against p-AMPKα, AMPKα, cleaved caspase-3, Bax, Bcl-2 and α-tubulin antibodies (Cell Signaling Technology, danvers, MA, USA). After PBS washing membranes were incubated for 2 h with horseradish peroxidase conjugated secondary antibodies at room temperature. Membranes were developed using a Western Bright enhanced chemiluminescent (ECL) kit (Advansta, Menlo Park, CA, USA) according to instructions from the manufacturer.

### *4.5. Statistical analysis*

The differences were analyzed between various groups using one-way analysis of variance and Tukey's post *hoc test*. Data from experiments are presented as the mean±standard error of triplicate experiments conducted independently. $P<0.05$ were taken to represent statistically significant differences. Statistical analysis of the data was performed using SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA).

Conflicts of interest: None declared.

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