Inhibition of osteosarcoma cell proliferation in vitro and tumor growth in vivo in mice model by alstonine through AMPK-activation and PGC-1α/TFAM up-regulation

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Osteosarcoma, a leading malignant tumor of bones is diagnosed mostly in adolescents and young adults worldwide. The present study investigated alstonine as an osteosarcoma agent in vitro as well as in vivo and evaluated the underlying mechanism. Treatment with alstonine led to a significant (P<0.05) reduction in MG63 and U-2OS cell viability. Alstonine treatment of MG63 and U-2OS cells caused a significant reduction in colony formation compared to the control cells. Viability of osteoblasts was not affected by alstonine treatment in 1.25 to 20 µM concentration range. In alstonine treated MG63 and U-2OS cells apoptotic cells increased significantly (P<0.05) compared to the control cells. Moreover, in MG63 and U-2OS cells treatment with alstonine caused a prominent increase in expression of cleaved caspase-9, caspase-3, and PARP. Treatment of MG63 and U-2OS cells with alstonine caused a prominent increase in AMPKα (Thr172) phosphorylation and elevated the count of mtDNA copies compared to the untreated cells. Alstonine treatment of the cells caused a remarkable increase in expression of PGC-1α and TFAM proteins. Treatment of the mice with alstonine at 5 and 10 mg/kg doses for 30 days caused a significant (P<0.05) reduction in xenograft growth. Expression of PGC-1α and TFAM proteins in tumor tissues of the mice treated with alstonine was significantly (P<0.05) raised compared to the control group. Thus, alstonine inhibits osteosarcoma cell growth and activates apoptosis through AMPK dependent pathway. Therefore, alstonine may be considered for treatment of osteosarcoma as it effectively arrests tumor growth in mice.

Keywords: alstonine, osteosarcoma, mitochondrial biogenesis, chemotherapy, alkaloids

INTRODUCTION

Osteosarcoma, a leading malignant tumor of bones is diagnosed mostly in adolescents and young adults worldwide. Surgical resection is the most effective therapeutic way used by clinicians to inhibit disease metastasis (Hegyi et al., 2011; Jaffe, 2009). Although, adjuvant chemotherapy has shown some improvement in osteosarcoma prognosis, during the last two decades desired results could not be obtained despite advancement in therapeutic interventions. During phase I/II clinical trials the most effective drugs against osteosarcoma have been found to induce high toxicity (Van Mallegem et al., 2012). This highlighted the immediate need for the development of new treatment strategies to inhibit osteosarcoma growth and metastasis.

Mitochondria, earlier only known for regulation of energy metabolism, have a key role in apoptotic process and are thereby used as therapeutic target in cancer treatment (Chan, 2006). Mitochondrial biogenesis and its dysfunction are regulated during several types of cancers using therapeutic agents as anticancer strategy (Neuzil et al., 2013). In some types of cancers including stomach cancer, breast cancer, renal carcinoma, and hepatocellular carcinoma the number of mitochondria has been found to be quantitatively reduced (Yamada et al., 2006; Yu et al., 2007; Wu et al., 2005; Lee et al., 2004; Xing et al., 2008; Tseng et al., 2006). On the other hand, the number of mitochondria is significantly raised in some of the kinds of cancers like ovary cancer, head and neck cancer, and oesophageal squamous cell carcinoma (Kim et al., 2004; Lin et al., 2010; Wang et al., 2006). Quantitative reduction in mitochondria have been demonstrated to be related with the patient prognosis and progression of tumor during breast carcinoma and hepatocellular cancer (Yamada et al., 2006; Yu et al., 2007). Mitochondrial count in sarcoma tissues of humans is significantly lower compared to that of the normal muscle cells (Onishi et al., 2014). Moreover, reduction in mitochondrial count during musculoskeletal malignancies has been demonstrated to play a key role in tumor progression (Onishi et al., 2014).

Transcription of the gene encoding mitochondrial transcription factor-A (TFAM) is regulated by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) during biogenesis of mitochondria (Ekstrand et al., 2004). The PGC-1α mediated transcription of TFAM has an important role in quantitative maintenance of mtDNA (Ekstrand et al., 2004). Increase in PGC-1α expression elevates mitochondrial numbers which subsequently induces sarcoma cell apoptosis through mitochondrial dependent pathway (Onishi et al., 2014).

Indole based compounds including various derivatives of indole (indole-3-carbinol, indole-3-carboxaldehyde), many functionalized indoles (diaryl-indoles, indolyl chal-
cones, indolyl azoles), and bisindoles have been found to act as potential anticancer agents (Dadashpour, 2018). Several reports have highlighted the role of various indole derivatives as effective cancer compounds (Leoni et al., 2016; Kumar et al., 2013; Sravanthi et al., 2016; Sunil et al., 2017; Chadha et al., 2017; El-Sayed et al., 2015). Indole alkaloids including vinblastine, vincristine, vin-camnine, and camptothecin isolated from the plants possess significant anti-cancer activity (Cordell et al., 2001). Alstonine, an indole alkaloid, is the major component isolated from several plant species and has been used in traditional medicine for treatment of mental illnesses (Costa-Campos et al., 1999). It has been found to prevent the development of many solid tumors and acts as an antineoplastic agent (Beljanski et al., 1986). The present study investigated alstonine as anti-osteosarcoma agent in vitro as well as in vivo and evaluated the under mechanism.

**MATERIALS AND METHODS**

**Cell lines**

Normal human osteoblasts and two osteosarcoma cell lines (MG63 and U-2OS) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All the cell lines were cultured for overnight in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) mixed with fetal bovine serum (FBS; 10%; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (100 U/ml penicillin/streptomycin; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were cultured in an incubator under humidified 5% CO₂ atmosphere at 37°C.

**Cell proliferation assay**

The cells were seeded in 96-well culture plates at 2×10⁴ cells/well density and incubated overnight at 37°C. Cells in three compound wells were used for each concentration to obtain triplicate values. The cells were treated with 1.25, 2.5, 5.0, 10, and 20 µM concentrations of alstonine for 72 h prior to removal of the culture solution. Then 10 µl MTT (5 mg/ml) was poured into each of the culture plate and cells were incubated for 4 h at 37°C. Removal of culture solution was followed by addition of 100 µl DMSO to each well for solubilization of the insoluble material formed. Optical density of the cell suspension was measured using enzyme labeling instrument (Sunrise; Tecan Group, Ltd.) at 575 nm to plot the survival curves.

**Colony formation assay**

Cell suspension aliquots containing 1000 cells was added to the 60-mm culture dishes and cells were cultured at 37°C under 5% CO₂ atmosphere. During cell culture, medium from the dishes was changed after every 3 days and finally removed to wash the cells using PBS. The cells were then fixed in 100% anhydrous methyl alcohol for 20 min at room temperature. Number of colonies having greater than 15 cells was counted in each dish using a light microscope (magnification, ×400; Carl Zeiss AG).

**Western blot analysis**

Cell lysates were prepared by treatment with RIPA lysis buffer (including 1 µM PMSF protease inhibitor) for 45 min on ice. Tumor samples obtained from the mice were also lysed on treatment with the RIPA lysis buffer. The lysate was centrifuged at 13,000×g for 20 min at 4°C to isolate the supernatant in which protein content was estimated using BCA reagent kits (Bio-Rad, Hercules, CA, USA). Protein samples (30 µM) were resolved by electrophoresis on 8–10% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and then transferred to PVDF membranes. The membranes were blocked on incubation with 5% skimmed milk powder for 2 h at room temperature. Incubation of the membranes was performed with primary antibodies at 4°C overnight. The antibodies used were against: PGC-1α (dilution 1:1000; Cat No. H00011089-M12), TFAM (dilution 1:1000; Cat No. H00007019-B01P) (both from Abnova, Walnut, CA, USA), p-AMPKα (Thr172; dilution 1:1000; Cat No. 2531S), AMPKα (dilution 1:1000; Cat No. 2532S) (both from Cell Signaling Technology, USA), PARP (dilution 1:1000; Cat No. 9542S), cleaved PARP (dilution 1:1000; 56258), caspase-3 (dilution 1:1000; Cat No. 9668S), cleaved caspase-3 (dilution 1:1000; Cat No. 9664S), caspase-9 (dilution 1:1000; Cat No. 9502S), cleaved caspase-9 (dilution 1:500; Cat No. 7237S; all from Cell Signaling Technology), and anti-human α-tubulin antibody (dilution 1:10,000; Cat No. T9026; Sigma-Aldrich). After incubation, membranes were washed and then incubated for 1.5 h with horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology) at room temperature. The protein bands were visualized using ECL Plus Western Blot Detection reagent system (GE Healthcare Biosciences, Piscataway, NJ, USA) and a Chemilumino Analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction (qPCR)**

Mitochondrial numbers in the cells were examined by determination of relative mtDNA amount to the total nuclear DNA (nDNA). The commercially available GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) was used for isolation of genomic DNA from the cells. Primer sequences used for the amplification of mtDNA were: sense 5'-GGAGATTTGGG-TACCAC CCAAGTATTGACTCACC-3' and anti-sense 5'-GCATGGAG AGCTCCCGTGA遣-GTGA-TAATAGGTTGATAG-3'. Conditions used for PCR were: one cycle at 94°C for 17 min, followed by 39 cycles at 94°C for 25 sec, 56°C for 25 sec, and 70°C for 80 sec. Relative quantity of mtDNA to nDNA in the cells was determined using the ΔΔCt method.

**Apoptosis analysis**

Apoptosis induction in the alstonine treated cells was detected using flow cytometric assay. The cells (at 2×10⁴ cells/ml density) from culture plates were treated with 1% paraformaldehyde in PBS and then resuspended in 1% ethyl alcohol (ice-cold). The cells were subjected to staining with Annexin V-FITC and PI double-dye for 15 min at 37°C. Cells were analyzed for induction of apoptosis by flow cytometry (BD FACSCalibur™; BD Biosciences). The data was evaluated using CellQuest Pro software (version 5.0; BD Biosciences).

**Animal studies**

Fifty Male BALB/c nude mice (6-weeks old; 25–35 g) were purchased from the Animal Center, Qinhuangdao Haigang Hospital, China. All the mice were maintained in animal house under pathogen-free conditions in the
plastic cages and acclimatized for 1-week before actual start of the experiment. Temperature was controlled at 23±1°C, humidity 65% and mice were given free access to laboratory chow and autoclaved water. The mice were exposed to 12-h light/dark cycles. Mice were assigned randomly into five groups: Normal, alstonine (alstonine + normal saline), Model (tumor), three alstonine-treatment (at 5, 10 and 20 mg/kg doses) groups and normal (sham) group. The U-2OS cells (2×10^5 in 500 μl PBS) were carefully implanted into the mice in model and two treatment groups dorsally in subcutaneous region to induce tumor development. The mice in alstonine-treatment groups received 5 and 10 mg/kg doses of alstonine in physiological saline through intragastric route. Body weight of the mice was measured alternately for 30 days after tumor cell inoculation. Tumor development was determined by measurement of tumor volume using the vernier callipers. The mice were sacrificed by cervical dislocation using 1% sodium pentobarbital (50 mg/kg) anaesthesia on day 31st to extract the tumors for western blotting experiment.

**Statistical analysis**

The data presented are the mean ± S.D. of triplicate measurements. All the data were analysed using the SPSS 20 (IBM Corp.) software. Data were compared between groups using one-way ANOVA followed by Bonferroni post hoc test. Differences were considered statistically significant at \( P<0.05 \).

**RESULTS**

**Alstonine affects MG63 and U-2OS cell viability**

Treatment with alstonine for 72 h led to a significant \( (P<0.05) \) reduction in MG63 and U-2OS cell viability in 1.25 to 20 µM concentration range (Fig. 1). Viability of MG63 and U-2OS cells showed a dose-dependent reduction on treatment with alstonine treatment at 1.25 to 20 µM doses. Alstonine treatment at 1.25 and 20 µM doses reduced MG63 cell viability to 93 and 27%, respectively compared to the control. Similarly, in U-2OS cells viability was suppressed to 95 and 32% on treatment with alstonine at 1.25 to 20 µM doses after 72 h, respectively. Moreover, alstonine treatment of MG63 and U-2OS cells at 1.25 to 20 µM doses caused a significant reduction in colony formation compared to the control cells. Viability of osteoblasts was not affected by alstonine treatment in 1.25 to 20 µM concentration range.

**Figure 1. Effect of alstonine on viability and colony formation in osteoblasts, MG63 and U-2OS cells.** (A) Cell viabilities were measured after 72 h of incubation with alstonine at 1.25 to 20 µM doses by MTT assay. (B) Number of colonies formed by the cells following incubation with 1.25 to 20 µM doses of alstonine was also calculated. *\( P<0.05 \) and **\( P<0.01 \) vs control (0 concentration) cells.

**Figure 2. Effect of alstonine on apoptosis induction in MG63 and U-2OS cells.** (A) Cell apoptosis was detected after 72 h of incubation with alstonine at 1.25 and 20 µM doses by flow cytometry. (B) Protein expression in the cells was assessed by western blotting following 72 h of incubation with 1.25 and 20 µM doses. Protein expression was normalized to α-tubulin. Where ‘0’ concentration stands for control.
Alstonine promotes MG63 and U-2OS cell apoptosis

In alstonine treated MG63 and U-2OS cells apoptosis induction was detected after 72 h by flow cytometry (Fig. 2A). Alstonine treatment of the cells at 1.25 to 20 µM significantly ($P<0.05$) raised percentage of apoptotic cells compared to the control cells. Moreover, in MG63 and U-2OS cells treatment with alstonine at 1.25 to 20 µM caused a prominent increase in expression of cleaved caspase-9, caspase-3, and PARP (Fig. 2B).

Alstonine promotes AMPK phosphorylation and increases mitochondrial proliferation

Treatment of MG63 and U-2OS cells with alstonine at 1.25 and 20 µM doses caused a prominent increase in AMPKα (Thr172) phosphorylation compared to the control (Fig. 3). Expression of phosphorylated AMPKα (Thr172) was higher in 20 µM alstonine treated MG63 and U-2OS cells compared to the 1.25 µM treated cells. Moreover, alstonine treatment of the cells for 72 h led to a significant ($P<0.05$) increase in count of mtDNA copies compared to the untreated cells.

Alstonine promotes PGC-1α/TFAM expression in osteosarcoma cells

In MG63 and U-2OS cells alstonine treatment for 72 h was followed by western blotting to determine changes in PGC-1α and TFAM expression (Fig. 4). Alstonine treatment of the cells at 1.25 and 20 µM for 72 h caused a remarkable increase in expression of PGC-1α and TFAM proteins. Again, increase in PGC-1α and TFAM expression was higher in MG63 and U-2OS cells on treatment with 20 µM alstonine compared to the 1.25 µM treated cells.

In vivo tumor growth suppression by alstonine

Osteosarcoma xenograft growth was significantly ($P<0.05$) higher in U-2OS cell implanted mice compared to the control group (Fig. 5). Treatment of the mice with alstonine at 2.5, 5, and 10 mg/kg doses for 30 days caused a significant ($P<0.05$) reduction in xenograft growth. Decrease in xenograft growth was higher in mice treated with 10 mg/kg dose compared to those administered with 2.5 and 5 mg/kg alstonine.

Alstonine promotes apoptosis in osteosarcoma xenografts

Alstonine treatment of the mice at 5 and 10 mg/kg doses significantly ($P<0.05$) increased apoptotic cell percentage in the osteosarcoma xenograft compared to the control group (Fig. 6A). Increase in cell apoptosis by alstonine treatment was higher in 10 mg/kg treated group in comparison to the mice treated with 5 mg/kg dose. Expression of PGC-1α and TFAM proteins in tumor tissues of the mice was significantly ($P<0.05$) lower compared to the control group (Fig. 6B). Treatment with alstonine at 5 and 10 mg/kg doses for 30 days caused a prominent elevation in PGC-1α and TFAM protein expression in mice tumor tissues.
Alstonine inhibits proliferation of osteosarcoma cells

DISCUSSION

Osteosarcoma patients with metastatic or recurrent stage of tumor have very poor prognosis because of non-availability of second-line chemotherapies (Friebele et al., 2015; Mirabello et al., 2009). Development of novel and more potential treatment strategies are required to inhibit the progression of high-grade osteosarcoma and improve the prognosis. In the present study alstonine treatment led to a significant (P<0.05) reduction in MG63 and U-2OS cell viability in dose dependent manner. Additionally, alstonine treatment of MG63 and U-2OS cells caused a significant reduction in colony formation compared to the control cells. Apoptosis induction was raised significantly in MG63 and U-2OS cells on treatment with alstonine for 72 h. Moreover, in MG63 and U-2OS cells treatment with alstonine caused a prominent increase in the expression of cleaved caspase-9, caspase-3, and PARP. Thus, initial findings showed cytotoxic potential of alstonine against MG63 and U-2OS cells in dose-dependent manner.

Mitochondrial involvement in death of cells via programmed manner is well established and it has been demonstrated that PGC-1α/TFAM pathway regulates mtDNA during biogenesis process (Chan et al., 2006; Ekstrand et al., 2004). Changes in mitochondrial numbers have been observed in human beings during several malignancies and studies have shown reduction in mitochondrial count to be involved in tumor progression (Yamada et al., 2006; Yu et al., 2007; Wu et al., 2005; Lee et al., 2004; Xing et al., 2008; Tseng et al., 2006). Thus, improvement in mitochondrial dysfunction is considered to be an important target for treatment of cancers (Neuzil et al., 2013). Reduction in mitochondrial count has been found in human sarcoma cells and it has been demonstrated that increasing mitochondrial number through PGC-1α expression upregulation results in apoptosis of the cells possessing lower mitochondrial count (Onishi et al., 2014). It is also reported that cell metabolism is related with cancer growth and an enzyme regulating energy, AMPK plays crucial role as kinase to control various checkpoints of the metabolic cell cycle (Sanhi et al., 2010). Additionally, AMPK also plays a key role in regulating mitochondrial biogenesis by direct activation of PGC-1α expression in skeletal muscles (Jäger et al., 2007). Activated AMPK caused tumor growth suppression by arresting cell cycle of induction of apoptosis after p53 and FOXO3a phosphorylation (Gwinn et al., 2008; Shackelford & Shaw, 2009). There are reports that mitochondrial biogenesis can be used as a therapeutic strategy for cancer treatment by activating AMPK/PGC-1α/TFAM axis on administration of various AMPK activators like metformin (Cheng et al., 2014; Guo et al., 2009; Sauve et al., 2012; Su et al., 2007; Woodward and Platania, 2010). Activation of AMPK has been demonstrated to promote transcription and replication of mtDNA in HeLa cells by increasing PGC-1α, NRF-1, and TFAM mRNA expression (Fu et al., 2008). Cell cycle arrest is mediated by AMPK activation through up-regulation of p21cip1 and p27kip1, which are the cyclin-dependent kinase inhibitors (Gwinn et al., 2008; Mihaylova and Shaw, 2011). The present study examined effect of alstonine on AMPKα (Thr172) phosphorylation in vitro in MG63 and U-2OS cells. It was observed that treatment of MG63 and U-2OS cells with alstonine caused a prominent increase in AMPKα (Thr172) phosphorylation compared to the control. Alstonine treatment of the cells also led to a significant (P<0.05) increase in count of mtDNA copies compared to the untreated cells. Moreover, in MG63 and U-2OS cells, alstonine treatment caused a remarkable increase in expression of PGC-1α and TFAM proteins. Increase in PGC-1α and TFAM expression was higher in MG63 and U-2OS cells on treatment with 20 µM alstonine compared to the 1.25 µM treated cells.
Thus, these findings indicated that alstonine induces mitochondrial apoptosis in MG63 and U-2OS cells by the up-regulation of PGC-1z expression via phosphorylation of AMPK.

In vivo studies were also in consistence with the results obtained under in vitro conditions on MG63 and U-2OS cells. It was observed that treatment of the mice with alstonine at 5 and 10 mg/kg doses for 30 days caused a significant (P<0.05) reduction in xenograft growth. Decrease in xenograft growth was higher in mice treated with 10 mg/kg dose compared to those administered with 5 mg/kg alstonine. Moreover, alstonine treatment of the mice significantly (P<0.05) increased apoptotic cell percentage in the osteosarcoma xenograft. Treatment with alstonine caused a prominent elevation in PGC-1z and TFAM protein expression in mice tumor tissues.

CONCLUSION

In summary, the present study demonstrated that alstonine treatment inhibited osteosarcoma cell viability by activation of apoptosis, AMPK activation mediated by PGC-1z/TFAM pathways. Moreover, osteosarcoma tumor growth was also inhibited in mice model by alstonine treatment through PGC-1z/TFAM pathway up-regulation and activation of AMPK phosphorylation. Thus, alstonine may be developed as a potential therapeutic agent for treatment of osteosarcoma.

Declarations

Acknowledgments. Not applicable.
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Competing interests. The authors state that there are no conflicts of interest to disclose.
Ethics approval. The study was approved by Animal Ethics Committee, Fifth People’s Hospital of Jinan, Shandong Jinan, China (Approval number PH/2018/0032). The study was performed in accordance with the guidelines issued by the National Institute of Health, USA (Guo et al., 2009).

Statement of Informed Consent. Not applicable.

Authors’ contributions. YB conceived and designed the study. QY, JJ, JX and SJ conducted the experimental work, analysed the data, and performed the literature survey. QY, and JX compiled that data and wrote the paper. All the authors approved the paper for publication.

Availability of data and materials. All data generated or analysed during this study are included in this published article.

REFERENCES


