Melatonin Increases the Sensitivity of Osteosarcoma Cells to Chemotherapy Drug Cisplatin

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ABSTRACT

Chemotherapy, which is one of the common treatments for osteosarcoma (OS), has many side effects and in some cases has low effectiveness due to chemoresistance, hence it is vital to study new therapies for OS. In this regard, we combined melatonin with cisplatin and evaluate their effect on MG63 OS cells. Since melatonin has anti-cancer properties, we hypothesized that its combination with cisplatin could increase the effectiveness of cisplatin. Firstly, MTT assay was used to evaluate the cell viability and cytotoxicity of cisplatin on MG63 cells and the results showed that melatonin in combination with cisplatin increases the sensitivity of MG63 cells to cisplatin. In addition, qRT-PCR results showed that the expressions of miR-181 and P53, CYLD, CBX7 and BCL2 genes change in MG63 cells after treatment with the combination of cisplatin and melatonin, so that the expression of P53, CYLD and CBX7 increased and the expression of BCL2 and miR-181b decreases significantly. Furthermore, analysis of Annexin V/FITC assay data revealed that the rate of apoptosis in MG63 OS cell line remarkably promoted after treated with cisplatin and melatonin combination. As a result, our findings show that melatonin in combination with cisplatin increases the effectiveness of cisplatin in osteosarcoma cells and this study provides a new therapeutic approach for OS.

Introduction

Osteosarcoma (OS) is a primary bone malignancy that affects long bones and mostly occurs in young people [1]. OS usually metastasizes to the lungs and it is a sign of poor prognosis in OS patients [2]. In 1970, the prevalent treatment of OS was amputation which was not effective on patients' survival rate, while in 1980, neoadjuvant chemotherapy and surgery to some extent increased patient survival, but the survival rate was still low in patients with metastasis [3]. Since chemotherapy and surgery have side effects and are invasive treatment methods, efforts are being made to discover new strategies in OS treatment.

Cisplatin (cis-diamminedichloroplatinum II, DDP) is a platinum-based chemotherapy drug which is used against a variety of cancers such as ovarian cancer, lung cancer, and OS. Cisplatin binds to DNA through N7 nucleophilic sites and inhibits the production of mRNA and protein, which ultimately prevents tumor cells proliferation and initiates apoptosis [4, 5]. Although cisplatin is widely used in chemotherapy, its side effects and resistance of OS cells to cisplatin are two of the most important issues that have led researchers to seek new therapies [6]. One of the novel strategies based on cisplatin is combinational therapy in which a low dose of cisplatin combines with other agents to improve its efficacy on tumor cells [6, 7].

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a physiological hormone that regulates the sleep-wake cycle in the human body. Furthermore, studies have proven that MLT plays an important role in cancer. MLT can be a pro-apoptotic and anti-metastatic agent in cancers including OS. Thus, it reduces tumor development and improves immune system activity. MLT can be used as a combination agent along with chemotherapy drugs to reduce their side effects and increase the efficacy of chemotherapy drugs in OS [8–10].

microRNAs (miRNAs) are small and non-coding RNAs (ncRNAs) which regulate gene expression through binding to 3'-UTR of mRNA [11]. miRNAs play an important role in cancers and usually act as suppressor tumors or oncogenes. Accordingly, they can be used as diagnostic or therapeutic, or prognostic biomarkers in cancers [12, 13]. miR-181 family includes four miRNAs: miR-181a, miR-181b, miR-181c, miR-181d. miR-181b upregulates in pancreatic and bladder cancer and OS while it downregulates in gastric and prostate cancer [11]. Upregulation of miR-181b increases OS proliferation and metastasis [14]. In some types of cancer miR-181b target some specific genes, for instance, it is shown that miR-181b targeted CBX7, and reciprocally CBX7 negatively regulated miR-181b in human breast adenocarcinoma cells [15]. Furthermore, CYLD is another potential target of miR-181b in breast cancer and thyroid papillary cancer. miR-181b contributes to cell growth in breast cancer and thyroid cancer by suppressing the expression of CYLD [16, 17].

BCL2 is an anti-apoptotic protein that prevents the release of cytochrome c from mitochondria, thereby inhibiting caspase and apoptosis activation. Hence, BCL2 increases tumorigenesis. High expression of BCL2 in OS is associated with poor prognosis and low survival [18–20].

P53 is a tumor suppressor gene that has several roles in cells such as growth arrest, apoptosis, and DNA stability. The expression of P53 is decreased in OS which is correlated with poor prognosis in patients and chemotherapy resistance. Low expression of P53 reduces apoptosis rate in OS cells. On the other hand, upregulation of P53 leads to chemosensitivity in OS [21, 22].

Materials and Methods

All experimental procedures were applied in accordance with the approval from the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1398.265).

Materials

The material used for cell culture included RPMI-1640 medium, fetal bovine serum (FBS), and trypsin solution. Chemical reagents included MLT (Cayman, USA), cisplatin (Cayman, USA). RT-PCR reagents included RNA extraction solution (Cinnagen), SYBR green master mix, and reverse transcriptase kit which were purchased from Bioneer company.

Cell Culture

For cell culture, the Human OS cell line MG63 was provided from the Pasteur Institute of Iran (Tehran, Iran). Saos2 was cultured in RPMI-1640 medium containing 10 % FBS and incubated at 37°C, 5 % CO2, and 95 % humidity. The culture medium is sterilized by 0.22 µ microbiological filters and stored at 4°C. The cells were passaged with wash solution (PBS) and 1 ml of trypsin.

Cell viability and proliferation assay

Cell proliferation was determined by MTT assay. MG63 cell line was seeded into 96-well plates (5×10^3 cells per well). The plate was divided into 4 groups: 1. Control without any treatment, 2. MLT, 3. cisplatin, 4. combination of MLT and cisplatin (50/50). The plate was incubated at 37°C with CO2 and 95 % humidity for 24–72 hours. After this period, the media were removed and washed with Phosphate Buffered Saline (PBS), and then MTT solution was added to each well then incubated for 4 hours. After 4 hours, 200 µL of DMSO was added to each well. The absorption was measured by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm.

Evaluation of cell apoptosis

10^6 of MG63 cells per well were seeded in 6 wells and treated with MLT, cisplatin, and a combination of them. The cells were detached with trypsin and centrifuged. Then, the cells were washed with 1 mL of PBS and 100 µL of binding buffer to remove trypsin. The MG63 cells were dissolved in FITC-Annexin V and incubated for 10 min and then 400 µL of binding buffer was added to cells. Finally, apoptosis was analyzed by flow-cytometer.

Quantitative RT-PCR

Total RNA was extracted from cells using Trizol reagent and its concentration was determined by NanoDrop. RNA was treated with DNAse and reverse transcribed with RT kit (Bioneer) for cDNA synthesis. Then, cDNA was quantified on real-time PCR detection system using SYBR Green PCR master mix. The expression levels of genes were measured based on the comparative C_t method ($2^{-}\Delta C_t$). The reverse and forward primer sequences are listed in ▶ **Table 1** (▶ **Table 1**).

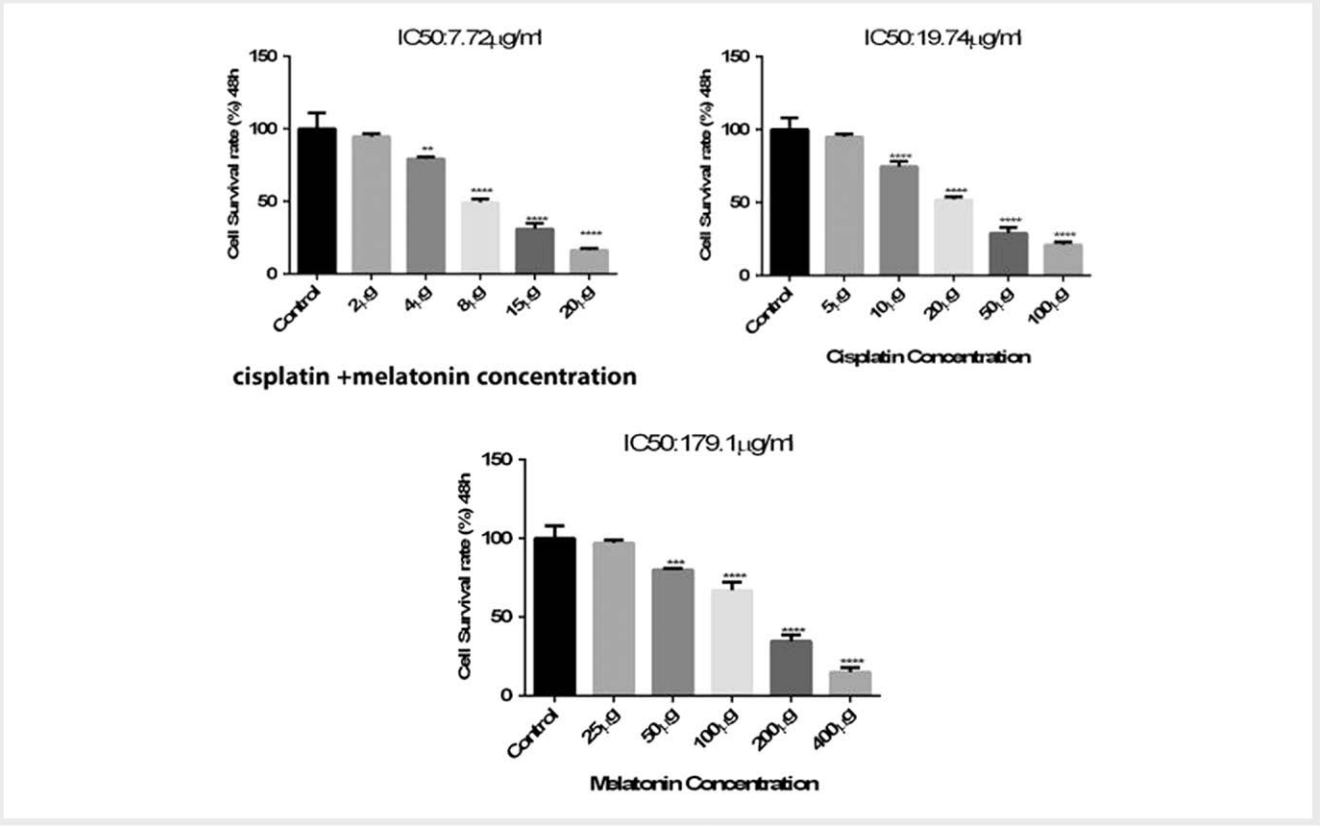
Results

The effect of melatonin on cytotoxicity of cisplatin in MG63 osteosarcoma cells

MTT assay has been used to evaluate the cytotoxic effect of melatonin, cisplatin, and their combination in different doses on MG63 OS cells. IC50 of each of the above drugs was measured. The IC50s obtained for cisplatin and melatonin and their combinations are 19.74 µg/ml, 179.1 µg/ml, and 7.72 µg/ml. After 48 h, the survival rate of MG63 OS cells was significantly reduced (▶ **Fig. 1**). IC50 of the combination of MLT and cisplatin diminished and this means that the sensitivity of MG63 OS cells to cisplatin has been improved

▶ **Table 1** PCR Primers Sequences.

Gene	Forward	Reverse	Annealing temperature
CBX7	CATGGAGCTGTCAGCCATC	CTGTACTTTGGGGGCCATC	59.0℃
BCL2	CCTCCAGGTAGGCCCGTTTT	GGGCCTCTGTTCCTTCCCTC	57.5℃
P53	GCGTGTGGAGTATTTGGATG	GTACAGTCAGAGCCAACCTC	61.0℃
CYLD	CCTTTATGTCAAGAGGTGGTG	GAGTAATGATTGGAAAGAAG	59.0℃
miR-181b	CGTGTATTTGACAAGCTG	GAACATGTCTGCGTATCTC	58.5℃



▶ **Fig. 1** cytotoxicity of cisplatin, MLT, and cisplatin-MLT combination on MG63 OS cells; the combination of cisplatin and MLT inhibited cell viability in low concentration.

(▶ **Fig. 1**). These results prove that MLT enhances the cytotoxic effect of cisplatin on OS cells.

Cisplatin-MLT combination downregulates expression of BCL2 and miR-181b

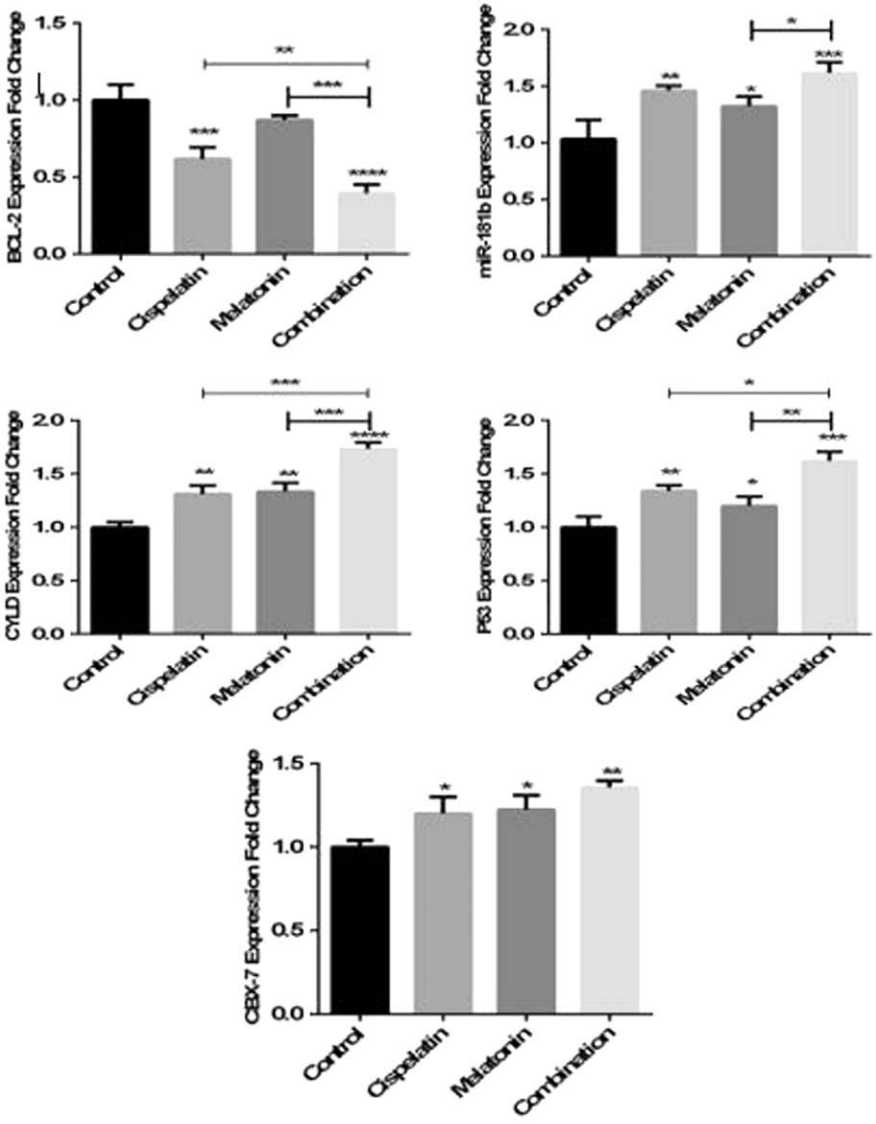
BCL2 and miR-181b play an oncogenic role and their expression increases in OS. Cisplatin notably reduces BCL2 expression while MLT slightly downregulates it. The combination of cisplatin and MLT significantly downregulates BCL2 expression (▶ **Fig. 2**). Cisplatin-MLT combination was expected to decrease the expression of miRNA-181b in the MG63 OS cell, however the data show that the combination of cisplatin-MLT increased miR-181b expression unexpectedly (▶ **Fig. 2**).

Cisplatin-MLT combination upregulates expression of CYLD, CBX-7, and p53

CYLD, CBX-7, and p53 act as tumor suppressors, and their expression reduce in OS. PCR results revealed that cisplatin and melatonin each promote the expressions of CYLD and CBX-7 slightly (▶ **Fig. 2**). Increased expression of p53 by cisplatin was slightly greater than the increased expression of p53 by MLT. Our results demonstrated that the cisplatin-MLT combination remarkably enhances the expression of these genes (▶ **Fig. 2**).

The effect of cisplatin-MLT on apoptosis

Flow cytometry results demonstrated that the rate of apoptosis in MG63 OS cells was increased meaningfully after treatment with cisplatin, MLT, and cisplatin-MLT compared to untreated cells. In ▶ **Fig. 3**, the cells are alive in the Q1-LL region and Annexin V $^-$ and PI $^-$, in the Q1-LR region, cells enter the early apoptosis phase, in which Annexin



▶ **Fig. 2** qRT-PCR results after 48 h treatment with cisplatin, MLT and cisplatin-MLT in MG63 cells. A) The expression level of oncogenic genes such as BCL2 and miR-181b decreases; B) the expression of tumor suppressor genes including CYLD, p53, CBX-7 increases.

V^+ and PI^- and in the Q1-UR region, cells are in the late stage of apoptosis and the cells die, where Annexin V^+ and PI^+. As shown in the ▶ **Fig. 3**, apoptosis increased after the cells were treated with a combination of MLT and cisplatin (▶ **Fig. 3**).

Statistical Analysis

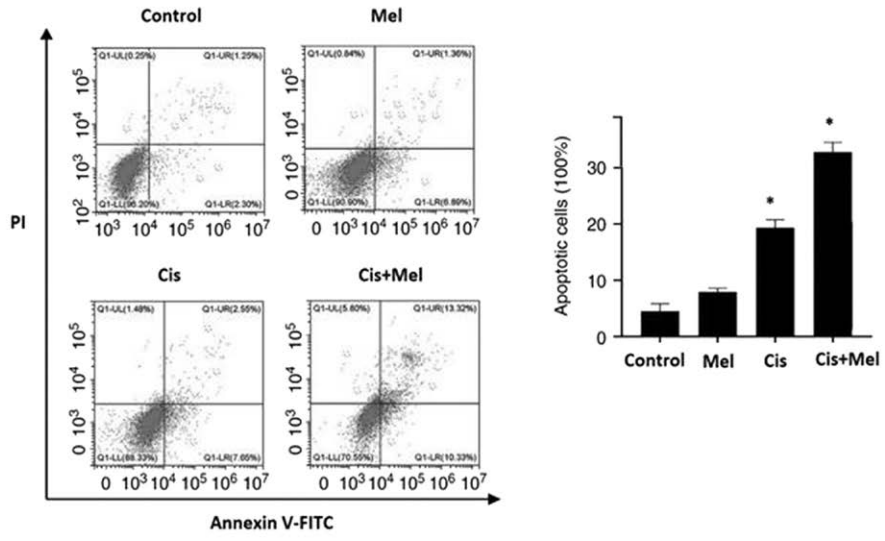
All experimental data were analyzed using Student t-test and SPSS software.

Discussion

Melatonin has an anti-cancer role in several cancers through its anti-oxidant, pro-apoptotic, anti-inflammatory and anti-angiogenic properties [9, 23]. In this study, we investigated that MLT can alleviate the resistance of MG-63 OS to cisplatin and in other words, MLT can increase chemosensitivity in OS. More studies need to confirm our results.

In recent years, common treatments for OS, including neoadjuvant chemotherapy and surgery, do not have acceptable outcomes in OS patients with metastasis [24, 25], it is vital to study new treatments strategies for osteosarcoma such as combination therapy and targeted therapy.

MLT plays different roles in cancers such as restoring drug sensitivity, inducing apoptosis, and inhibiting cell growth and metastasis, therefore it is a hormone as well as a cell protector and can be utilized as a natural agent in OS treatment [26, 27]. It is identified that melatonin inhibits the proliferation and invasion of osteosarcoma cells in a dose-dependent manner. It also inhibits VEGFA and angiogenesis by targeting miR-424–5p [26, 28].



▶ **Fig. 3** Apoptosis rate in MG63 OS cells treated with cisplatin, MLT, and cisplatin-MLT; apoptosis rate of MG63 cells increases after treatment with cisplatin-MLT and more cells enter the early apoptosis and death phase.

The combination of MLT with chemotherapy drugs increases their effectiveness and reduces the resistance of cancer cells to them. The combination of MLT with chemotherapy drugs, such as cisplatin or methotrexate, increases the survival rate of normal cells while decreasing the viability of cancer cells [29]. In this regard, Mir et al. proposed that MLT in combination with doxorubicin (DOX) decreases the survival of MG63 and Saos2 OS cells and also promotes the therapeutic effect of DOX on OS cells through downregulating the expression of X-linked Inhibitor of Apoptosis (XIAP) and Survivin [30].

miRNAs are associated with tumorigenesis and their expression is altered in cancers [12, 31]. They play a role in drug sensitivity and metastasis by regulating signaling pathways or regulating their target genes involved in cancers [32]. the expression of miR-181b is dysregulated in various cancers and it is one of the important miRNAs in cancers progression [31, 33]. It is identified that low expression of miR-181b is associated with cisplatin resistance in small cell lung cancer cells and its upregulation inhibits this resistance by downregulation of Bcl2 [31]. Furthermore, the expression of miR-181b upregulates in OS, and its high expression increases proliferation and metastasis while reducing the apoptosis rate in OS cells. According to previous studies, miR-181b regulates apoptosis and invasion in MG63 and U2OS cells by targeting p53 [13].

Bcl2 is an antiapoptotic protein that is altered in cancers. Bcl2 inhibits apoptosis in cancers cells by removing the pro-apoptotic and inducing anti-apoptotic genes [34]. Bcl2 is an ideal agent for targeting therapies and combination therapies in cancers [35]. Chemotherapy drugs including cisplatin induce DNA damage which release cytochrome C. Release of cytochrome C activates caspase-9 and consequently activates caspase-3 [36, 37]. Since Bcl2 is the substrate of caspase-3, caspase-3 cleaves Bcl2 at 34th amino acid and deactivates it. The product of this cleavage acts as a pro-apoptotic protein [36]. Therefore, cisplatin downregulates Bcl2 expression indirectly through regulating caspase-3. MLT promotes apoptosis by activating MAPK pathway. In this mechanism, MLT induces phosphorylation of JNK, ERK and p38, consequently increases caspase 3 cleavage an Bax expression ond reduces the expression of Bcl2 [38, 39]. In addition, in mitochondrial- depend pathway, increased Bax translocation to mitochondria by MLT, enhances mitochondrial membrane permeability and caspase activation, and eventually decreases Bcl2 expression [39]. MLT in combination with cisplatin induce apoptosis through downregulating Bcl2 in HepG2 hepatocellular carcinoma cells [40]. According to Xiyue Zhang et al. study, miR-181b-5p targeted Bcl2 by binding to its 3'-UTR and downregulated its expression. Moreover, the combination of mir-181b-5p and temozolomide also reduces the expression of Bcl2 in glioma cells [41].

p53 is a tumor suppressor gene that induces apoptosis by inducing cell cycle arrest and also determines the cytotoxicity of chemotherapeutic drugs in cancer and its upregulation promotes the sensitivity of OS cells to chemotherapeutic agents [42, 43]. As mentioned earlier, cisplatin interferes with N7 purine bases in DNA and forms inter-strand and intra-strand crosslinks which can inhibit the cell cycle and activate p53 and apoptosis [44, 45]. MLT reduces antioxidant defense in cancer cells and produces ROS which can increase p53 protein expression and apoptosis [46]. Moreover, according to another study, MLT promotes P53 to phosphorylation at serine 15, thereby inhibits cell proliferation and DNA damage in transformed cells [47]. Bennukul et al. demonstrated that melatonin along with cisplatin increase apoptosis and decreases oxidative stress by targeting and regulating p53 in hepatocellular carcinoma cells [40].

CBX7 mostly acts as a tumor suppressor whose low expression is associated with poor prognosis in various cancers and is involved in cancer progression by regulating EMT and drug resistance related genes. In this regard, Rong Li et al. identified that CBX7 down-

regulates in cervical cancer, and high expression of CBX7 increases apoptosis and reduces proliferation in cervical cancer [48].

CYLD is a deubiquitination enzyme and plays tumor-suppressive role in malignancies whose expression is downregulated in several cancers [16, 49]. In addition, CYLD is a negative regulator of the NF-KB signaling pathway and studies have shown that CYLD promotes the sensitivity of cells to chemotherapy agents by inhibiting the activity of the NF-KB pathway in gastric cancer [50].

Conclusion

In this study, we identified that MLT improve the cytotoxic effect of cisplatin, which is one of the common therapeutic agents in cancers, on OS cancer cells. Our findings show that the combination of MLT and cisplatin promotes the apoptosis rate and the expression of tumor suppressive genes including CBX7, CYLD and p53, while this combination reduces survival of cancer cells and suppresses the expression of oncogenic genes such as Bcl2 and miR-181b in MG63 OS cell line. Taken together, these results suggest that MLT lead to chemo-sensitizing OS cells to cisplatin and can be considered as an enhancer agent of the therapeutic effect of chemotherapy drugs on cancers.

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Conflict of Interest

The authors declare that they have no conflict of interest with the contents of this article.

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