

Targeted Antiosteosarcoma Methotrexate-bisphosphonate Conjugate induces apoptosis of osteosarcoma cells *in vitro*

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Abstract. – OBJECTIVES: To investigate the effect of targeted antiosteosarcoma methotrexate-bisphosphonate conjugate on growth inhibition and apoptosis in human osteosarcoma MG-63 cells.

MATERIALS AND METHODS: MG-63 cells were treated with various concentrations of methotrexate-bisphosphonate conjugate and apoptosis was monitored via an MTT assay, cell morphology, TUNEL assay and flow cytometry analysis.

RESULTS: The survival rate of MG-63 cells treated for 24 to 96 hours with 2000 µg/ml or more of methotrexate-bisphosphonate conjugate decreased significantly. Cells treated with conjugate showed typical apoptotic features using inverted phase contrast microscopy and fluorescence staining, and the majority of cells demonstrated a positive result in the TUNEL assay. Karyopyknosis and crescent aggregation of chromatin were observed in conjugate-treated cells by electron microscopy. Flow cytometry of MG-63 cells treated with methotrexate-bisphosphonate conjugate showed a time and dose-dependent increase in apoptosis ($p < 0.05$).

CONCLUSIONS: A targeted antiosteosarcoma methotrexate-bisphosphonate conjugate induces apoptosis in human osteosarcoma MG-63 cells. This new conjugate is a valuable experimental tool for the therapy of osteosarcoma.

Key words:

Methotrexate, Conjugate, Osteosarcoma, Apoptosis, Bisphosphonate.

the local concentration and duration of action of chemotherapeutic agents, how to minimize the systemic adverse reactions and side effects induced by chemotherapy, and how to reduce the frequency of administration of chemotherapy while improving the efficacy of the agents. Based on the characteristic molecular structure, methotrexate was coupled with a bone targeting bisphosphonate resulting in the synthesis of a methotrexate-bisphosphonate conjugate (hereinafter referred to as the conjugate) with bone target tropism *in vitro* as described previously¹. An ideal targeted conjugate shall maintain both characteristics of target tropism and cytotoxicity of chemotherapeutics². In the present study, a human osteosarcoma MG-63 cell line was chosen as a model to investigate the conjugate for its effect on growth inhibition and apoptosis induction as monitored by cell morphology, flow cytometry, and TUNEL assay. We show that the conjugate induces apoptosis and significantly reduces the survival rate of MG-63 cells. These results suggest that the conjugate is a valuable candidate for chemotherapeutic development.

Materials and Methods

Materials

The methotrexate-bisphosphonate conjugate was synthesized by the West China School of Pharmacy, Sichuan University. The human osteosarcoma MG-63 cell line was purchased from the Immuno-genetic laboratory of the West China Hospital of Sichuan University. Trypsin, tetramethyl azo salt (MTT), acridine orange and propidium iodide (PI) were products of Sigma Resources and Technologies Inc. (Santa Clara, CA, USA). DMEM media was purchased from Gibco (Carlsbad, CA, USA). Phase contrast im-

Introduction

Primary and metastatic osteosarcomas are among the most common malignant orthopedic diseases. However, several issues remain to be resolved including how to effectively maximize

ages were collected on an inverted phase contrast microscope CK40 (Olympus, Tokyo, Japan). Flow cytometry was performed on an Elite SP flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence microscopy was performed on a BH2 fluorescent microscope (Olympus, Tokyo, Japan). Electron microscopy was performed on an H600-IV transmission electron microscope (Hitachi, Tokyo, Japan).

Preparation of conjugate

One hundred milligrams of synthesized conjugate were dissolved in DMEM media supplemented with 10% fetal bovine serum (FBS). After filtration, the conjugate was diluted to a range of concentrations including 3000 µg/ml, 2000 µg/ml, 200 µg/ml, 20 µg/ml and 2 µg/ml and stored sterilely.

Cell culture

Human osteosarcoma MG-63 cells were thawed and recovered. The cells were cultured in medium containing 10% FBS in an incubator with 5% CO₂ at 37°C. Only cells that were in log phase were selected for the experiments.

MTT Assays

Cells were seeded in a 96 well plate at a density of 2×10^5 cells per well. After 24 hours, the cells were treated with the conjugate at a range of concentrations including 3000 µg/ml, 2000 µg/ml, 200 µg/ml, 20 µg/ml and 2 µg/ml and methotrexate (MTX) alone at concentration of 1500 µg/ml. Eight replicates were performed for each condition and also for an untreated control. Data were collected at 24hr, 48hr, 72hr and 96hr after treatment, respectively. Twenty microliters of MTT solution was added to each well at concentration of 5 mg/ml. The supernatant was discarded after 4 hours of incubation. Then, 150 µl dimethyl sulfoxide (DMSO) was added and the absorbance of each sample was measured at 490 nm using a microplate reader (BioTek, Burlington, NC, USA).

Cell morphology study

Human osteosarcoma MG-63 cells that were treated with different concentrations of the conjugate and untreated controls were observed with an inverted phase contrast microscope. These samples were also stained with acridine orange and analyzed by fluorescence microscopy. Osteosarcoma cells treated with 2000 µg/ml conjugate for 48hr were collected, centrifuged and

fixed with 1% osmium tetroxide. Then, samples were cut into ultrathin sections with a microtome, which were stained with lead citrate and observed using transmission electron microscopy.

TUNEL assay

One milliliter of MG-63 osteosarcoma cell suspension (cell density of 2×10^5 /ml) was added to a 6-well chamber slide and treated with the conjugate at a concentration of 2000 µg/ml for 48hr. The samples were washed, fixed and TUNEL analysis was performed following the manufacturer's instructions (Roche Diagnostic Corporation, Basel, Switzerland).

Flow cytometric analysis

Osteosarcoma cells were cultured in the absence or presence of 2000 µg/ml conjugate or 1500 µg/ml methotrexate separately for 24, 48, 72 and 96 hours. At the end of treatment, treated and control cells (with no drug added) were trypsinized with 0.25% trypsin, centrifuged, washed, fixed and stained with propidium iodide (PI) and analyzed by flow cytometry. The rate of apoptosis for each sample was expressed as mean $\pm$ standard deviation.

Statistical analysis

Statistical analysis was performed using a software package (SPSS 13.0, SPSS Inc., Chicago, IL, USA). Data are presented as means $\pm$ standard deviation. All analysis was performed using Pearson's chi-squared test and $p < 0.05$ was considered statistically significant.

RESULTS

Treatment with conjugate inhibits growth of human osteosarcoma MG-63 cells

We treated MG-63 osteosarcoma cells with concentrations of conjugate ranging from 0 µg/ml to 3000 µg/ml, or with 1500 µg/ml of MTX for 24, 48, 72 and 96 hours and observed their growth in culture (Figure 1). As shown in Figure 1, cultures treated with 200 µg/ml or more conjugate showed a decline in cell growth, and treatment with 2000 µg/ml or more conjugate or 1500 µg/ml MTX resulted in cell death. This downward trend in the growth curve could indicate cell apoptosis or cell death.

Figure 1. Growth curves of osteosarcoma MG-63 cells treated with various concentrations of conjugate or MTX. MG-63 cells were treated with 0 $\mu\text{g/ml}$ (negative), 2 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$, 2000 $\mu\text{g/ml}$, 3000 $\mu\text{g/ml}$ conjugate, or 1500 $\mu\text{g/ml}$ methotrexate (MTX) for the times indicated. A blank control containing no cells is also shown (blank).

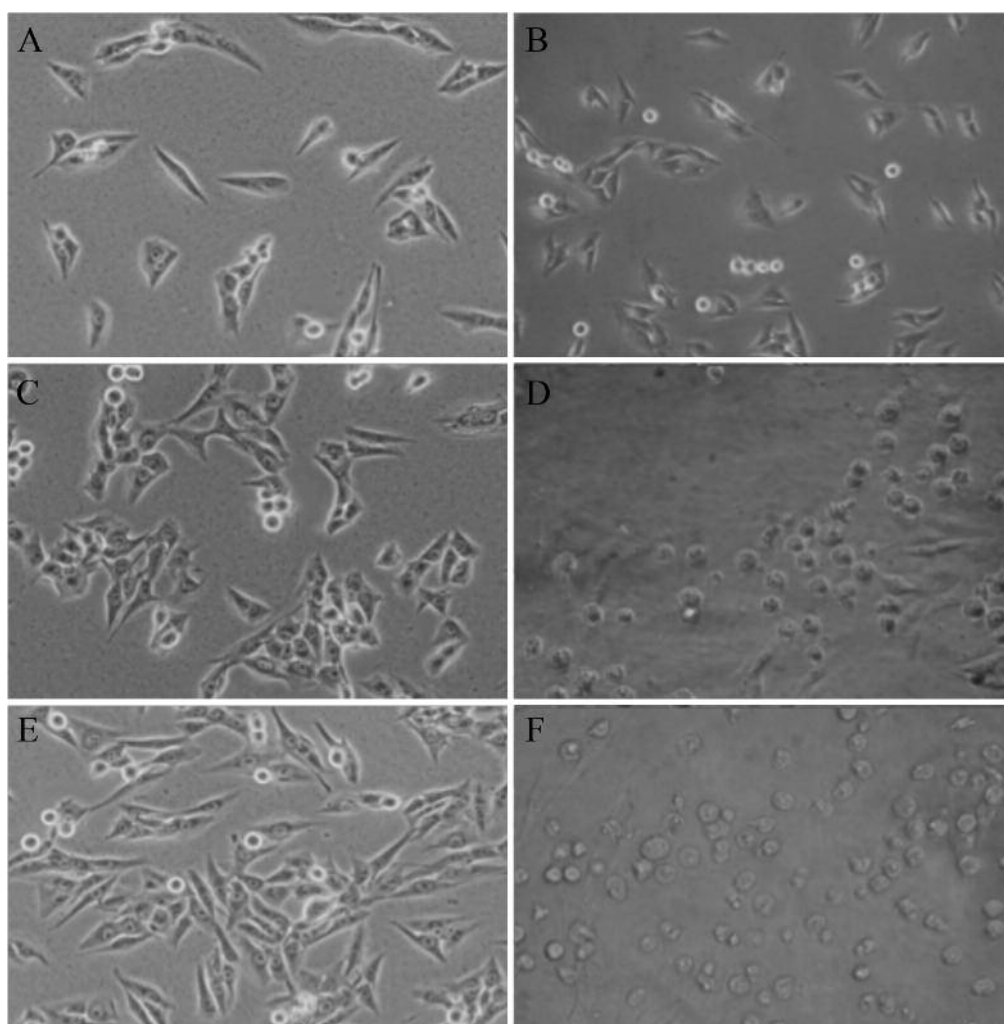
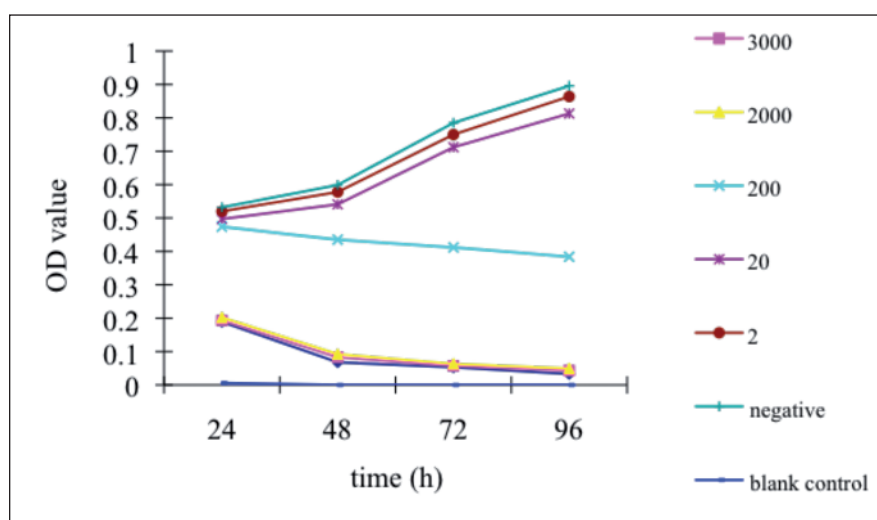


Figure 2. Treatment with high concentrations of conjugate changes the morphology of MG-63 cells. **A**, Control cells cultured for 24h. **B**, Cells treated with 2000 $\mu\text{g/ml}$ of conjugate for 24h. **C**, Control cells cultured for 48h. **D**, Cells treated with 2000 $\mu\text{g/ml}$ of conjugate for 48h. **E**, Control cells cultured for 96h. **F** Cells treated with 2000 $\mu\text{g/ml}$ of conjugate for 96h. All panels shown are at 200x magnification.

Treatment with high concentrations of conjugate changes the morphology of MG-63 cells

We studied the morphology of the cells treated with conjugate using inverted phase contrast microscopy. As shown in Figure 2, control cells grew well and were translucent and polygonal in shape (Figure 2A, C, E). In contrast, MG-63 cells treated with 2000 $\mu\text{g/ml}$ of the conjugate for 24h were round and smaller and sometimes had vesicles (Figure 2B). After 48h of treatment, the number of round cells and detached cells increased (Figure 2D). Furthermore cell necrosis and cell debris could be observed in some cells. After 96h of treatment, more cells underwent cell death and detached from the surface (Figure 2F). However, no significant changes in morphology were observed in cells treated with 200 $\mu\text{g/ml}$ of conjugate for the same time course (data not shown).

We next used acridine orange staining to assess the effects of the conjugate on the nucleus. Acridine orange bound nuclear DNA emits yellow or green fluorescence, while RNA in the cytoplasm and the nucleolus displays orange-red fluorescence. Acridine orange staining showed that in control cells, the chromatin is uniformly distributed

in the nuclei, as expected (Figure 3A). With increasing concentrations of conjugate and duration of treatment, cell size decreased, the nucleus condensed and dark yellow-green staining could be seen in nucleus (Figure 3B-C). Condensed, marginalized or even yellow-green stained fragmented chromatin was also observed (Figure 3B-C). In addition, plasma membrane protrusions and apoptotic bodies were observed (Figure 3B-C, *arrows*).

We used transmission electron microscopy to examine the features of the nucleus in both untreated cells and those treated with the conjugate. As shown in Figure 4A, in control cells, the nucleus was intact and regularly shaped with evenly distributed chromatin and prominent nucleoli. Abundant organelles including mitochondria could also be observed in cells with an intact plasma membrane. In contrast, with increasing concentration and duration of conjugate treatment, we observed more typical apoptotic cells displaying an empty nucleus, dilatation of rough endoplasmic reticulum, and condensed or marginalized chromatin (Figure 4B-C). We also observed multinucleated cells that had densely packed chromatin in crescent-shaped aggregates accumulating on the inner surface of the nuclear membrane (Figure 4B, *arrow*).

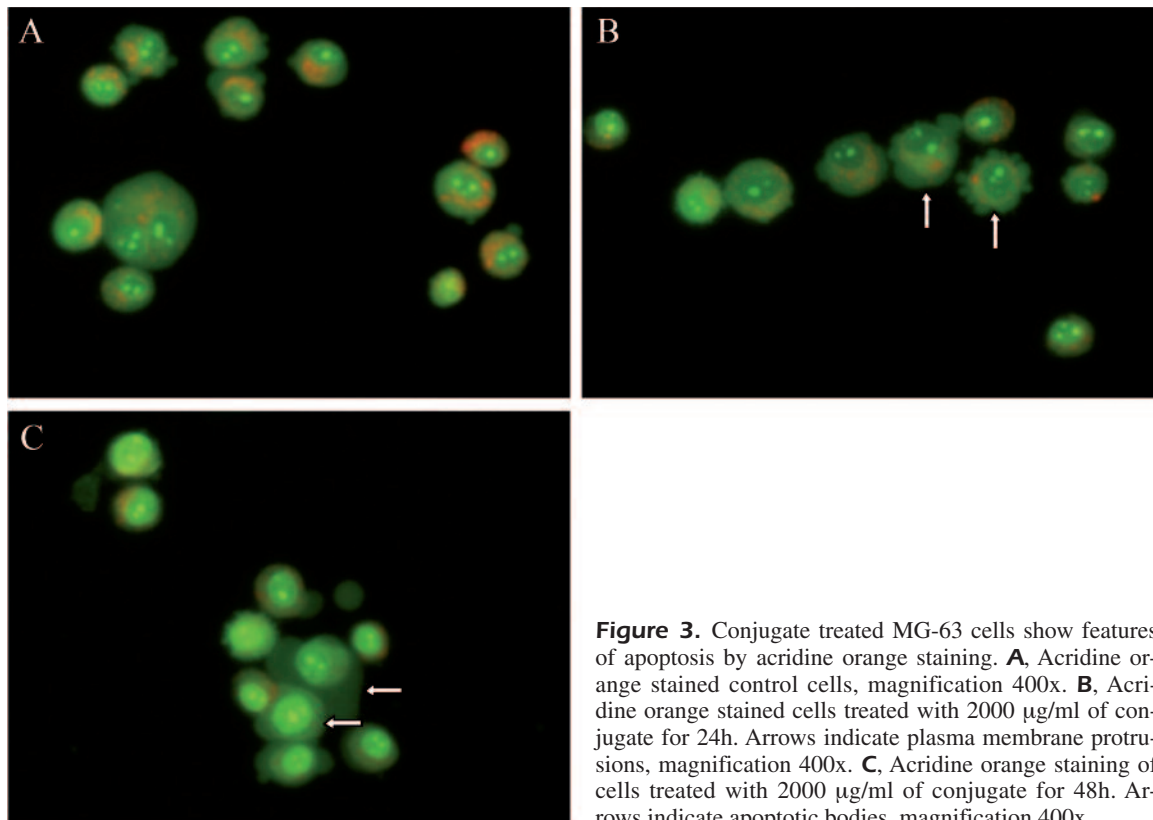


Figure 3. Conjugate treated MG-63 cells show features of apoptosis by acridine orange staining. **A**, Acridine orange stained control cells, magnification 400x. **B**, Acridine orange stained cells treated with 2000 $\mu\text{g/ml}$ of conjugate for 24h. Arrows indicate plasma membrane protrusions, magnification 400x. **C**, Acridine orange staining of cells treated with 2000 $\mu\text{g/ml}$ of conjugate for 48h. Arrows indicate apoptotic bodies, magnification 400x.

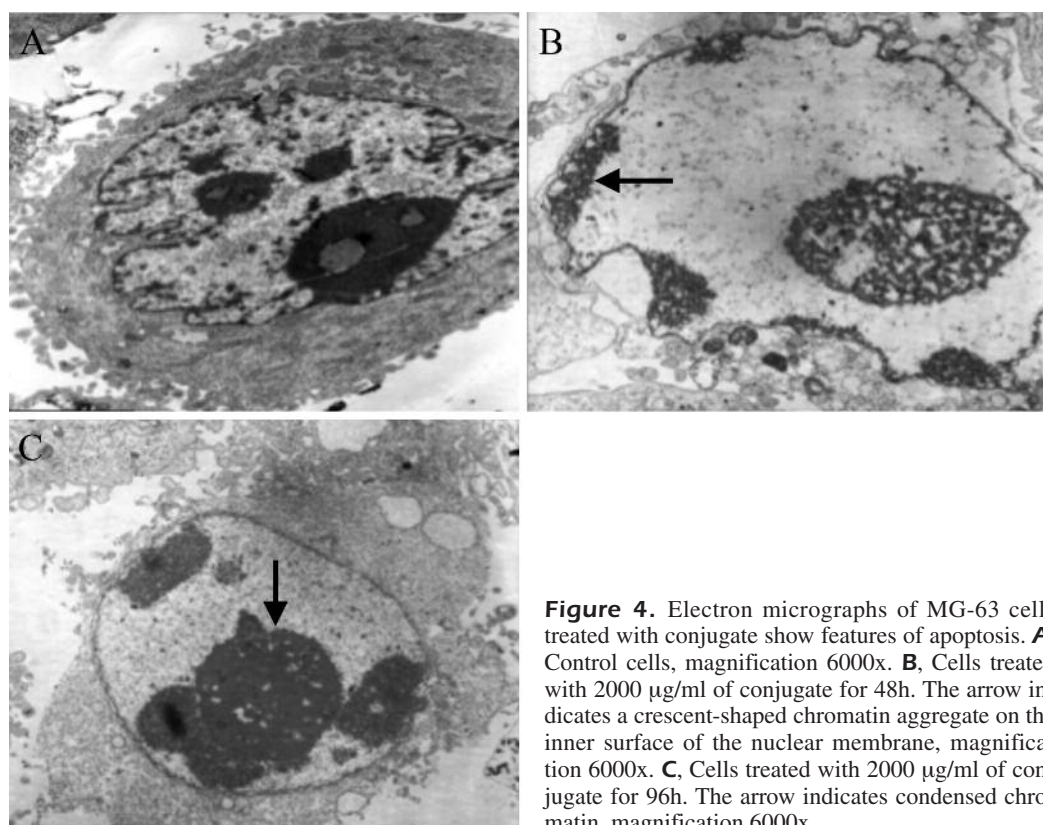


Figure 4. Electron micrographs of MG-63 cells treated with conjugate show features of apoptosis. **A**, Control cells, magnification 6000x. **B**, Cells treated with 2000 µg/ml of conjugate for 48h. The arrow indicates a crescent-shaped chromatin aggregate on the inner surface of the nuclear membrane, magnification 6000x. **C**, Cells treated with 2000 µg/ml of conjugate for 96h. The arrow indicates condensed chromatin, magnification 6000x.

MG-63 cells treated with conjugate show a significant hypodiploid peak

We used flow cytometry to determine whether the DNA in cells treated with conjugate was fragmented, as is characteristic of apoptotic cells. As shown in Table I, the apoptotic rates of cells treated with 2000 µg/ml of conjugate after 24h, 48h, 72h and 96h were 12.64%, 20.71%, 31.44% and 43.58%, respectively ($p < 0.05$). The apoptotic rate was positively correlated with the duration of drug treatment.

MG-63 cells treated with conjugate undergo apoptosis

We performed a TUNEL assay to directly monitor whether the cell death we observed after conjugate treatment was due to apoptosis. As shown in Figure 5, most MG-63 cells treated

with 2000 µg/ml of conjugate for 48 h underwent apoptosis (Figure 5B), whereas control cells did not show significant amounts of apoptosis (Figure 5A). We also observed the characteristic green fluorescence emitted by apoptotic cells in the samples treated with conjugate (Figure 5C).

Taken together, our data indicate that osteosarcoma cells treated with the conjugate undergo apoptosis. These data suggest that the conjugate could be a valuable tool for future chemotherapeutics.

DISCUSSION

An imbalance between apoptosis and cell proliferation is believed to result in tumor formation. Some studies have suggested the existence of a dual signaling module in which the induction of

Table I. The apoptotic rate of MG-63 osteosarcoma cells.

Treatment/ incubation time	24h	48h	72h	96h
Untreated	1.23±0.22	1.57±0.41	2.33±0.63	3.47±0.52
Conjugate (2000 µg/ml)	12.64±1.05	20.71±1.87	31.44±2.55	43.58±3.11
MTX alone (1500 µg/ml)	13.21±1.28	21.22±2.25	33.86±3.67	46.74±3.18

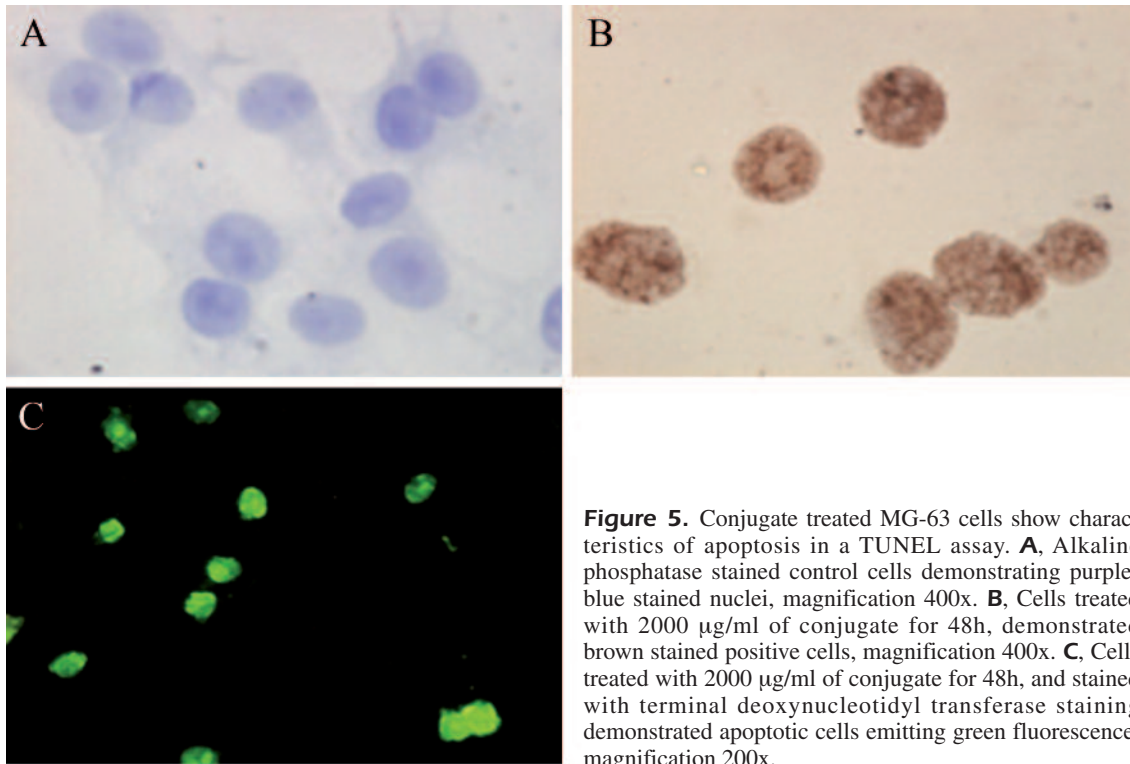


Figure 5. Conjugate treated MG-63 cells show characteristics of apoptosis in a TUNEL assay. **A**, Alkaline phosphatase stained control cells demonstrating purple-blue stained nuclei, magnification 400x. **B**, Cells treated with 2000 µg/ml of conjugate for 48h, demonstrated brown stained positive cells, magnification 400x. **C**, Cells treated with 2000 µg/ml of conjugate for 48h, and stained with terminal deoxynucleotidyl transferase staining demonstrated apoptotic cells emitting green fluorescence, magnification 200x.

proliferative signal pathways by oncogenes also leads to the activation of an apoptosis pathway³. In this model, cells can successfully proliferate only when the apoptosis signals are suppressed³. Therefore, the dysregulation of apoptosis plays a significant role in the development of malignant tumors. Inhibition of cell proliferation or activation of cell apoptosis is the key to the prevention and management of cancer. Apoptosis is a programmed cell death induced by multiple genes and signaling pathways and it is closely associated with the genesis, development and treatment of tumors. Studies have shown that the management of cancer is mainly achieved through the induction of cancer cell apoptosis by chemotherapeutic agents, and the sensitivity of cancer cells to chemotherapeutics determines the efficacy of the treatment⁴. Accordingly, the ability to induce apoptosis is considered one of the standards for screening antitumor agents⁵.

MTX is an effective chemotherapeutic agent in the treatment of several tumors including osteosarcoma⁶. It interferes with DNA and RNA synthesis in the S phase of the cell cycle and delays the G1-S phase transition through the inhibition of dihydrofolate reductase, resulting in the inhibition or arrest of cell division and leading to

apoptosis of cancer cells⁶. In this study, we show that a new therapeutic agent designed by the conjugation of MTX with bone-targeting diphosphate effectively inhibited proliferation and induced apoptosis of human osteosarcoma MG-63 cells.

Our morphological analyses show that increasing conjugate concentration and treatment duration results in an increase in cells with apoptotic features including round cells, enhanced refraction and the detachment of cells from the culture surface (Figure 2). A few studies have demonstrated that nuclear alterations occur after cell injury⁷⁻⁸. Multinucleated cells induced by injury are an early feature of apoptosis⁷. The formation of multinucleated cells is associated with the abnormal nuclear division of injured cells due to asymmetric separation of chromosomes which are enveloped by the smooth endoplasmic reticulum. In this study, cells treated with conjugate had crescent shaped chromosomes that were attached to the nuclear membrane (Figure 4), which is consistent with early features of apoptosis. We also observed cytoplasmic condensation and karyopyknosis in some cells treated with conjugate. In addition, we also found myelin bodies in the cytoplasm and the dilatation of the rough endoplasmic reticulum which appears frequently in dam-

aged or aging cells. Therefore, the morphological features of the cells treated with conjugate are indicative of significant cytotoxicity and apoptosis.

We showed that the conjugate effectively inhibits proliferation of osteosarcoma cells. Our results also demonstrated that no significant inhibitory effect was observed between 2000 µg/ml and 3000 µg/ml of conjugate and 1500 µg/ml MTX alone during the same period of time ($p > 0.05$). The fact that the conjugate was weaker than MTX may be related to trace impurities introduced during its synthesis.

The quantification of DNA by flow cytometry is one of the most common methods for the study of apoptosis. A typical flow cytometric marker of apoptosis is the appearance of a hypodiploid peak. The underlining mechanism includes the fragmentation of chromatin into oligonucleotides and DNA loss during the cell fixation and staining process, as well as a reduced affinity for the fluorescent probe caused by DNA condensation in apoptotic cells⁹. In the present study, we show that the apoptotic rate gradually increased with increasing duration of the treatment with conjugate (Table I), indicating that the conjugate induces apoptosis in a time-dependent and dose-dependent manner. The apoptotic rates of cells treated with 2000 µg/ml of conjugate after 24h, 48h, 72h and 96h were 12.64%, 20.71%, 31.44% and 43.58%, respectively. This concentration of conjugate is roughly equivalent to 1500 µg/ml of MTX, which suggests that the conjugate retains the characteristics of the original drug and acquires a significant apoptosis inducing effect as well.

DNA fragmentation by the activation of an endogenous endonuclease is a hallmark of apoptosis⁹. The fragmentation of DNA generates the same number of 3'-hydroxyl termini as the number of DNA fragments. Essentially, the TUNEL assay labels of the 3'-hydroxyl DNA ends generated during DNA fragmentation by means of a terminal deoxyribonucleotidyl transferase (TdT) and labeled dUTP. In the present study, we show that osteosarcoma cells treated with 2000 µg/ml of conjugate exhibit a large number of yellow-brown stained apoptotic cells; however, we observed significantly less apoptosis with either 200 µg/ml or 20 µg/ml of conjugate. In addition, apoptosis increased with increasing duration of conjugate treatment.

Apoptosis is a complex physiological process. Compared with necrotic cells, apoptotic cells demonstrate a series of specific features includ-

ing morphological changes such as cell shrinkage, cytoplasmic condensation, expansion of endoplasmic reticulum turning cytoplasm into vacuole, nuclear condensation or disintegration resulting in dispersion in the cytoplasm, chromatin condensation and marginalization, and the formation of an apoptotic body¹⁰. The DNA in apoptotic cells is degraded into multiple oligonucleotide fragments by caspase activated DNase (CAD) and other Mg^{2+} or Ca^{2+} dependent endonucleases, which results in the appearance of a DNA ladder in gel electrophoresis, whereas the DNA in necrotic cells is degraded randomly and appears as diffuse bands in electrophoresis¹⁰. Apoptotic cells also have increased expression of the glutathione transferase gene, higher activity of cathepsin D, tissue plasminogen activator and proteases relevant to the degradation of cytoskeleton¹⁰. Several experiments exist to distinguish necrosis from apoptosis, including morphological analysis, flow cytometry, TUNEL assay and others. Morphological analysis is the most objective method and electron microscopy enables the most detail to be observed. Nevertheless, no single method is foolproof. The best strategy is to use a combination of different methods to achieve more reliable results. In this study, we show that the conjugate induces changes in cells consistent with apoptosis by morphological analysis (Figures 2 to 4), by flow cytometry (Table I), and by TUNEL assay (Figure 5).

Conclusions

We demonstrate that the conjugate exhibits a significant apoptosis-inducing effect on osteosarcoma MG-63 cells in a time-dependent and dose-dependent manner. This conjugate may lead to the development of osteosarcoma targeting chemotherapeutics.

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Conflict of interest

The Authors declare that they have no conflict of interests.

References

- 1) YANG XN, PEI FX, ZENG JC. Effect of target antios-
teosarcoma methotrexate-bisphosphonates con-
jugate on human osteoblast in vitro. *West China*
Med J 2008; 23: 1086-1088.
- 2) WANG LF, ZHOU Y, XU YM, QIU XC, ZHOU BG, WANG
F, LONG H, CHEN X, YANG TT, MA BA, FAN QY, YANG
AG. A caspase-6 and anti-HER2 antibody
chimeric tumor-targeted proapoptotic molecule
decreased metastasis of human osteosarcoma.
Cancer Invest 2009; 27: 774-780.
- 3) PATINO-GARCIA A, ZALACAIN M, FOLIO C, ZANDUETA C,
SIERRASESUMAGA L, SAN JULIAN M, TOLEDO G, DE LAS
RIVAS J, LECANDA F. Profiling of chemonaive os-
teosarcoma and paired-normal cells identifies
EBF2 as a mediator of osteoprotegerin inhibition
to tumor necrosis factor-related apoptosis-induc-
ing ligand-induced apoptosis. *Clin Cancer Res*
2009; 15: 5082-5091.
- 4) AKIYAMA T, DASS CR, CHOONG PF. Novel therapeutic
strategy for osteosarcoma targeting osteoclast
differentiation, bone-resorbing activity, and apop-
tosis pathway. *Mol Cancer Ther* 2008; 7: 3461-
3469.
- 5) YANG R, PIPERDI S, GORLICK R. Activation of the
RAF/mitogen-activated protein/extracellular sig-
nal-regulated kinase kinase/extracellular signal-
regulated kinase pathway mediates apoptosis in-
duced by chelerythrine in osteosarcoma. *Clin*
Cancer Res 2008; 14: 6396-6404.
- 6) FROMIGUE O, HAMIDOUCHE Z, MARIE PJ. Blockade of
the Rhoa-JNK-c-Jun-MMP2 cascade by atorvas-
tatin reduces osteosarcoma cell invasion. *J Biol*
Chem 2008; 283: 30549-30556.
- 7) CHEN JT, FONG YC, LI TM, LIU JF, HSU CW, CHANG
CS, TANG CH. DDTD, an isoflavone derivative, in-
duces cell apoptosis through the reactive oxygen
species/apoptosis signal-regulating kinase 1
pathway in human osteosarcoma cells. *Eur J*
Pharmacol 2008; 597: 19-26.
- 8) WANG Z, ZHOU JY, KANAKAPALLI D, BUCK S, WU GS,
RAVINDRANATH Y. High level of mitogen-activated
protein kinase phosphatase-1 expression is asso-
ciated with cisplatin resistance in osteosarcoma.
Pediatr Blood Cancer 2008; 51: 754-759.
- 9) ZAMILPA R, RUPAIMOOLE R, PHELIX CF, SOMARAKI-CORMI-
ER M, HASKINS W, ASMIS R, LeBARON RG. C-terminal
fragment of transforming growth factor beta-in-
duced protein (TGFBIp) is required for apoptosis
in human osteosarcoma cells. *Matrix Biol* 2009;
28: 347-353.
- 10) ZHANG L, YU B, LIN JH. [Apoptosis induction of tra-
ditional chinese herb pianzihuang ([chinese char-
acters: see text]) in human osteosarcoma u-20s
cells]. *Zhongguo Gu Shang* 2009; 22: 265-268.