Arch Biol Sci. 2017;69(4):577-583 https://doi.org/10.2298/ABS160712001W

Reduced cell proliferation and promotion of apoptosis by Leu5AMD in osteosarcoma cells

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Received: July 12, 2016; **Revised**: December 2, 2016; **Accepted**: December 2, 2016; **Published online**: August 9, 2017

Abstract: Leu[5]AMD is an actinomycin D (AMD) analog obtained by replacing both N-methylvalines present in AMD with N-methylleucines. The potential of Leu[5]AMD for the treatment of osteosarcoma cells and its mechanism of action are not clearly understood. In this study, we examined the potential use of Leu[5]AMD in halting the proliferation of the human osteosarcoma cell line Saos-2. Saos-2 cells were treated with different concentrations of Leu[5]AMD, and proliferation, cell viability, apoptosis and the cell cycle were examined. Among the various concentrations used, 1.2 μM and 6 μM Leu[5]AMD exhibited a significant effect on Saos-2 cells compared with untreated cells. The proliferation assay and Hoechst staining indicated that Leu[5]AMD treatment of Saos-2 human osteosarcoma cells resulted in a significant reduction in cell proliferation and enhanced apoptosis. Western blot analysis revealed that Leu[5]AMD-treated cells had significantly reduced cyclin expression compared with the control. Leu[5]AMD is responsible for inducing cell cycle arrest and apoptosis. These results support the development of a new drug for the treatment of osteosarcoma.

Key words*:* Osteosarcoma; chemotherapy; Leu[5]AMD; apoptosis; cancer

INTRODUCTION

Osteosarcoma is one of the most common bone tumors in children and adolescents. It is described as a heterogeneous tumor affecting proximal and lateral bones [1,2] and is identified by its highly malicious effects and rapid progression [3]. An abnormal regulation of cell maturation and a deregulation of apoptosis are commonly observed in cancer cells and play an important role in tumor growth [4,5]. The inhibition of cancer cell growth or the induction of apoptosis is possible using current anticancer compounds available in the market [6,7]. However, several treatment methods have failed to treat osteosarcoma, mainly due to chemotherapeutic drug resistance [8], revealing the urgency for more efficient treatment methods using pro-apoptotic elements capable of inducing a quick response in osteosarcoma cells toward cytotoxic anticancer drugs.

Actinomycin D (AMD) is a widely-known antibiotic obtained from *Streptomyces parvulus* or *Streptomyces melanochromogenes*. AMD has a planar structure with 2-aminophenoxazin-3-one as a chromophoric group and two cyclic pentapeptide lactones as bulky groups. AMD, a chromopeptide, exhibits different biochemical and pharmacological properties [9,10]. It has been used for the treatment of several types of cancer [11-13]. Notably, it has been used for the treatment of cancers, including Wilms' tumor, and for gestational trophoblastic disease [11,12,14]. At present, AMD is clinically used as an anticancer agent or in combination with another drug to treat high-risk tumors [15-17].

AMD derivatives can be obtained synthetically by various methods, such as biosynthesis and total synthesis [18-20], to diminish its cytotoxicity on normal cells while simultaneously maintaining most of its anticancer properties. The modification of AMD has received increasing interest among scientists, as it can increase the selectivity against some cancers [20, 21]. Previous studies aimed to replace the amino acid residue present in AMD depsipeptides but have

failed. The reason for this is that the replacements cause inactivity or decreased activity; thus far, very few analogs have shown positive results. The formation of N-methylalanine from N-methylvalines serves as a good example to support the feasibility of the replacement process. Furthermore, this causes loss of antitumor ability and a considerable reduction in antimicrobial characteristics of AMD [22]. The highest degree of antitumor potential demonstrated *in vitro* resulted from N-methylleucine replacement [23]. The compound, known as Leu^{5}AMD, displays potential that is equal to that displayed by AMD, even at a 100-fold-lower dilution [23]. To determine the properties of Leu^{5}AMD in anticancer treatment for osteosarcoma, we examined its inhibitory effects on the proliferation of Saos-2 cells.

MATERIALS AND METHODS

Materials

The AMD analog Leu^{5}AMD was synthesized according to a slightly modified version of the procedure described in the literature [23]. Thin layer chromatography (TLC) was used to examine the product homogeneity using silica gel plates. Leu^{5}AMD formation was confirmed using nuclear magnetic resonance (NMR) and mass spectrometry methods. The chemical structure of Leu^{5}AMD is shown in Fig. 1. All antibodies (cyclin-A, D1, E and cleaved caspase-3) were purchased from Cell Signaling Technology (Danvers, MA, USA). A Hoechst kit was obtained from Beyotime Biotechnology Company (Shanghai, China). Anti-β actin was purchased from Abcam (Cambridge, UK). All other solvents and chemicals used were of high grade and did not require further purification.

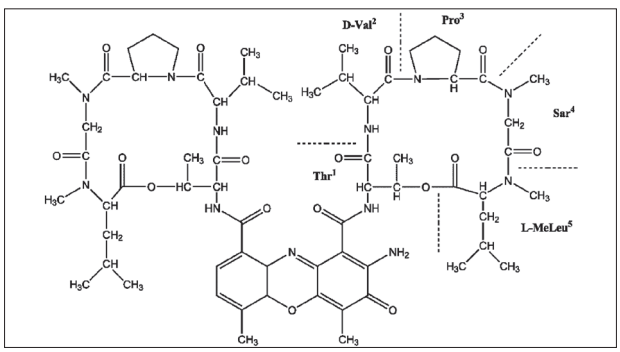

Fig. 1. Chemical structure of Leu^{5}AMD.

Cell lines and cell culture methods

The osteosarcoma cell line Saos-2 was purchased from the Shanghai Cell Institute, Chinese Academy of Science. Cells were grown in Dulbecco's modified essential medium (DMEM; Sigma-Aldrich, St. Louis, MI, USA) supplemented with penicillin (50 U/mL) (Sigma-Aldrich), streptomycin (50 μg/mL; Sigma-Aldrich) and fetal bovine serum (FBS, 10%; Beijing Minhai Biotech, Daxing, Beijing, China) and maintained at 37°C in humidified air with 5% CO_2.

For Hoechst staining, cells were plated onto 96-well plates at a density of 1x10^6 cells/mL. For Western blot analysis and reverse transcription polymerase chain reaction (RT-PCR), a cell density of 1x10^5 cells/mL was used [24]. In all studies, various concentrations (0, 0.1, 0.6, 1.2 and 6 μM) of Leu^{5}AMD were added to Saos-2 cells and incubated for 0, 4, 8 and 24 h before subsequent analyses. Ethanol was used as a solvent control.

Cell proliferation

The sulforhodamine B (SRB) colorimetric assay (Sigma-Aldrich) was used to examine cell proliferation [25]. In brief, cells were seeded onto 96-well plates at a density of 1x10^4 cells/well and treated with 0-6 μM Leu^{5}AMD. Cells were fixed using trichloroacetic acid (10%; Sigma-Aldrich) for 1 h at 4°C, washed, and stained with SRB (0.1%) dissolved in acetic acid (1%) for approximately 20 min at room temperature, followed by air drying. Bound SRB dye was dissolved in 50 μL of unbuffered Tris base solution (10 mM; Sigma-Aldrich) for approximately 30 min. Absorbance was measured at 490 nm using a microplate reader (Sunrise, Tecan Trading AG, Switzerland).

MTT assay and Hoechst staining

Cell viability was measured by the MTT assay. Cells, at a density of 1x10^4 cells/well, were seeded onto 96-well plates and incubated for 24 h. After incubation, cells were treated with 0-6 μM Leu^{5}AMD. Cell viability was measured by staining with 20 μL MTT solution (Sigma-Aldrich). After incubation at 37°C, the medium was removed, and dimethyl sulfoxide (DMSO; 150 μL) was added to solubilize the formazan. The

absorbance was recorded using a microplate reader, and cell viability was measured relative to the control. For Hoechst staining, Saos-2 osteosarcoma cells were directly stained using a Hoechst staining kit following the manufacturer's instructions (Beyotime Biotechnology Company, Shanghai, China). Cell counts were measured using ImageJ software.

Western blot analysis

Cells were lysed with lysis buffer containing phosphate-buffered saline (PBS) with Triton X-100 (1%; Sigma-Aldrich) and protease inhibitors. The supernatants from the cell lysates were harvested by centrifugation at 10000 rpm for 10 min at 4°C. Bio-Rad Protein Assay Reagent (Hercules, CA, USA) was used to measure the protein concentrations in the cell supernatants. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA), and incubated with the respective primary and secondary antibodies. Proteins were detected with SuperSignal Enhanced Chemiluminescence (ECL) Reagent (Merck Millipore, Billerica, MA, USA). Quantification was performed using ImageJ software.

Real-time PCR

TRI reagent (Sigma-Aldrich) was used for total RNA extraction. Reverse transcription of the RNA was performed using Maxima Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The Bio-Rad Miniopticon qPCR system with CFX Manager software (Hercules, CA, USA) was used for quantitative real-time PCR. Relative gene expression was normalized to that of β-actin, which served as a control. The primers were used as targets of SYBR green probes. Their sequences are as follows: *Cyclin A*, sense: AGCAGAAGAGAC TCAGAAAGG and antisense: GATA GTCAAGAGGTGTC-AGTGG; *Cyclin D1,* sense: CCGTCCAT GCGGAAGAC and antisense: ATCCAGCGGGAAGAC; *Cyclin E,* sense: GGAAG AGGAAG-GCAAACGTGA and antisense: TCGATTTTGGCCAT TTCTTCAT; *β-actin,* sense: GAGACCTTCAACACCCCAGC and antisense: ATGT CACGCACGATTTCCC.

Statistical analysis

Image software was used to perform the statistical analyses for all experiments. Quantitative data are represented as the means±standard error (SE) and were compared using analysis of variance (ANOVA). The following were used to indicate the levels of statistical significance: ***P value <0.001, **P value <0.01, and *P value <0.05.

RESULTS

Leu^{5}AMD inhibits proliferation of Saos-2 cells

To test the anticancer properties of Leu^{5}AMD against Saos-2 osteosarcoma cells, we studied the cell proliferation of Leu^{5}AMD-treated cells. The SRB colorimetric assay was used to measure cell proliferation under excellent growth conditions for 24 h.

In a preliminary experiment, the half-maximal inhibitory concentration (IC_{50}) of AMD and Leu^{5}AMD were compared and determined to be 1.6 μM and 0.7 μM, respectively (data not shown). A 6-μM concentration of Leu^{5}AMD exhibited a stronger inhibitory effect, whereas 1.2 μM Leu^{5}AMD only moderately inhibited cell proliferation. Other concentrations of Leu^{5}AMD, such as 0.1 μM and 0.6 μM, showed only a very minor effect on cell proliferation (Fig. 2). These

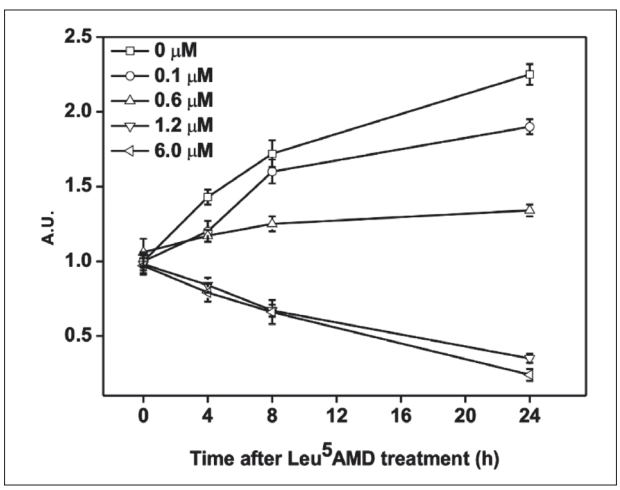

Fig. 2. Proliferation of Saos-2 osteosarcoma cells following Leu^{5}AMD treatment by a calorimetric assay. The results are given as the means±standard deviation (SD) of three independent experiments.

results suggest that Leu^{5}AMD inhibits the proliferation of Saos-2 human osteosarcoma cells in a time- and concentration-dependent manner.

Leu^{5}AMD induces apoptosis

Based on the inhibitory effects of Leu^{5}AMD on the proliferation of Saos-2 human osteosarcoma cells, we further determined whether Leu^{5}AMD induced apoptosis in Saos-2 cells. Hoescht staining was performed by treating Saos-2 cells with 6 μM Leu^{5}AMD for 4-24 h. At the tested concentration, Leu^{5}AMD induced apoptosis. The percentage of apoptosis increased with time, from 22.8% (4 h) to 54.4% (24 h) (Fig. 3A and 3B). To further confirm the role of Leu^{5}AMD on Saos-2 cell apoptosis, we measured the cell viability of Saos-2 cells after treatment with Leu^{5}AMD. Cell viability was reduced to 88% (4 h), 72.4% (8 h), and 42.9% (24 h) after treatment (Fig. 3C). Leu^{5}AMD induced apoptosis in Saos-2 cells in a time-dependent manner; hence, we decided to monitor the concentration-dependent effects of Leu^{5}AMD. According to Hoechst staining, the percentage of cellular apoptosis increased with increasing concentrations of Leu^{5}AMD (Fig. 4A and 4B). Additionally, the measured viable cell count was reduced upon Leu^{5}AMD treatment (Fig. 4C). These results indicate that Leu^{5}AMD induces apoptosis in Saos-2 cells in both a time- and a concentration-dependent manner.

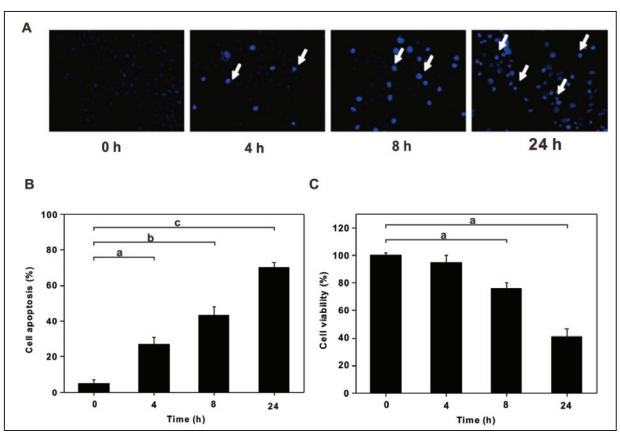

Fig. 3. Time-dependent induction of apoptosis in Saos-2 cells following Leu^{5}AMD treatment. The percentage of Leu^{5}AMD-treated Saos-2 cells that died is shown by Hoechst staining (**A**) and as a histogram (**B**). Cell viability (**C**) was reduced after 24 h treatment with Leu^{5}AMD. The data represent the means±SD of three independent experiments. **a** represents p<0.05, vs. the control at 0 h; **b** represents p<0.005, vs. the control at 0 h; **c** represents p<0.001, vs. the control at 0 h.

To further confirm the role of Leu^{5}AMD in apoptosis, we examined the expression of apoptotic markers using various concentrations of Leu^{5}AMD. Leu^{5}AMD activated caspase-3 in Saos-2 cells in a concentration-dependent manner (Fig. 5A and 5B).

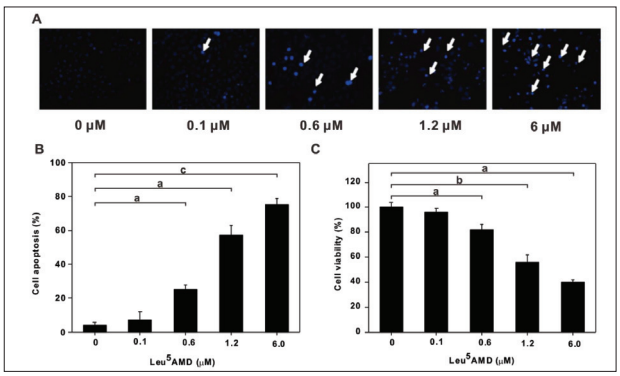

Fig. 4. Concentration-dependent induction of apoptosis in Saos-2 cells following Leu^{5}AMD treatment (0, 0.1, 0.6, 1.2 and 6 μM), as shown by Hoechst staining (**A**) and as a histogram (**B**). Saos-2 cell viability (**C**) was reduced after treatment with 0-6 μM Leu^{5}AMD for 24 h. The data represent the average of three independent experiments. Error bars indicate standard deviation. **a** represents p<0.05, vs. the untreated control; **b** represents p<0.005, vs. the untreated control; **c** represents p<0.001, vs. the untreated control.

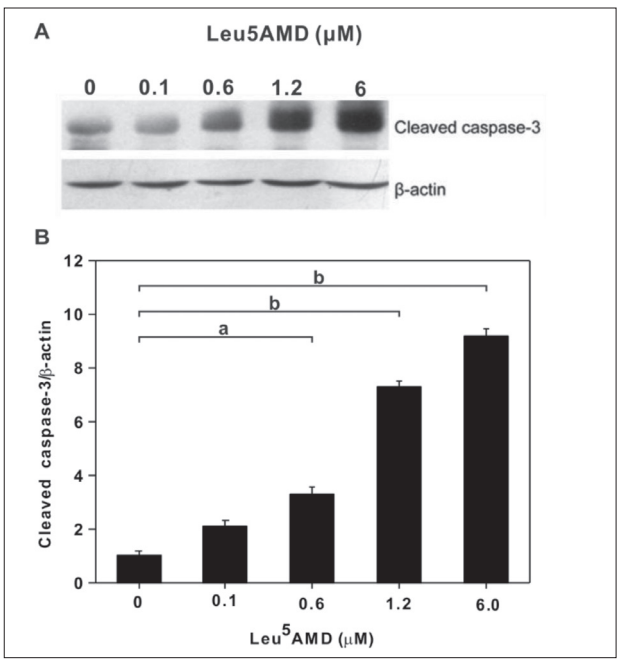

Fig. 5. Western blot analysis (**A**) and a histogram (**B**) showing the cleavage of caspase-3 protein in Saos-2 cells following treatment with 0-6 μM Leu^{5}AMD for 24 h. The data represent the means±SD of three independent experiments. **a** represents p<0.05, vs. the untreated control; **b** represents p<0.005, vs. the untreated control.

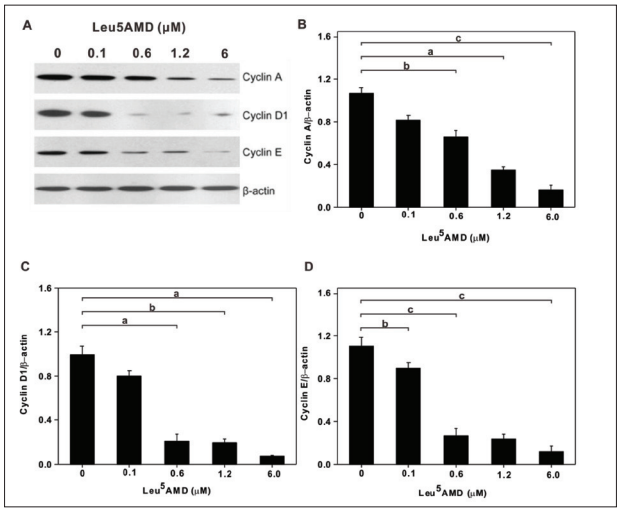

Fig. 6. Western blot analysis (**A**) and a histogram showing the expression of cyclin A (**B**), cyclin D1 (**C**), and cyclin E (**D**) in Saos-2 cells following treatment with 0-6 μM Leu^{5}AMD for 24 h. The results are given as the means±SD of three independent experiments. **a** represents $p<0.05$, vs. the untreated control; **b** represents $p<0.005$, vs. the untreated control; **c** represents $p<0.001$, vs. the untreated control.

Leu^{5}AMD reduces cyclin expression

To investigate in detail Leu^{5}AMD-induced cell proliferation and apoptosis at the molecular level in Saos-2 cells, we examined elements of the cell cycle. We believe that Leu^{5}AMD treatment reduces the protein expression of cyclins. To prove this hypothesis, we investigated changes in the protein levels of cyclin A, cyclin D1 and cyclin E. Leu^{5}AMD treatment significantly reduced the levels of all proteins examined (cyclin A, cyclin D1, and cyclin E). The effect was particularly marked in cyclin D1 (Fig. 6). As Leu^{5}AMD acts as an inhibitor of transcription, we assumed that Leu^{5}AMD affects cyclin protein levels through the inhibition of gene transcription. Real-time PCR was used to analyze the mRNA levels of cyclins upon Leu^{5}AMD treatment. The obtained data suggest that Leu^{5}AMD treatment reduces the mRNA levels of all cyclins. A parallel between changes in transcription levels and quantitative changes in the assayed cyclin protein levels was also observed (Fig. 7). These results indicate that Leu^{5}AMD downregulates the expression of cyclin proteins, in part via transcriptional regulation, and that it induces cell cycle arrest and apoptosis in Saos-2 human osteosarcoma cells.

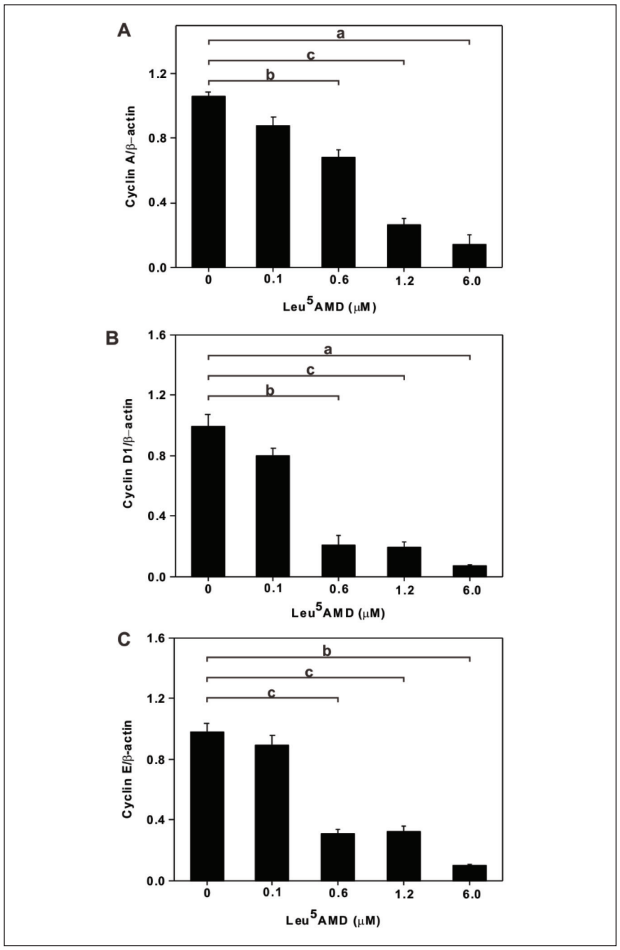

Fig. 7. Histograms showing decreased transcription of cyclin genes in Saos-2 cells following treatment with 0-6 M Leu^{5}AMD for 24 h. The mRNA levels of cyclin A (**A**), cyclin D1 (**B**), and cyclin E (**C**) decreased following treatment. The results are given as the means±SD of three independent experiments. a represents $p<0.05$, vs. the untreated control; b represents $p<0.005$, vs. the untreated control; **c** represents $p<0.001$, vs. the untreated control.

DISCUSSION

Many reports have shown that replaced amino acid components in AMD fail to exhibit potent antitumor effects against various cancer cells, whereas one previous study showed that Leu^{5}AMD, an AMD analog, exhibited comparatively greater antitumor effects than AMD alone [23]. Therefore, in this report, we aimed to determine whether Leu^{5}AMD inhibits the proliferation of Saos-2 human osteosarcoma cells.

One hallmark of cancer is uncontrolled cell proliferation; the corresponding rate is used to diagnose different types of cancer, including osteosarcoma

[26,27]. Thus, the inhibition of cell proliferation can be a useful platform for the development of cancer therapies. Based on this, we demonstrated that Leu^{5}AMD hinders the proliferation of and induces apoptosis in Saos-2 human osteosarcoma cells. Furthermore, these inhibitory effects are partially based on cyclin protein expression, as Leu^{5}AMD treatment hinders either cyclin gene transcription or protein translation. The downregulation of cyclin protein expression upon Leu^{5}AMD treatment is the reason for the cell cycle arrest and apoptosis induction, as shown in Fig. 7. These results strongly support a mechanism by which Leu^{5}AMD hinders Saos-2 osteosarcoma cells and induces apoptosis, therefore possibly serving as a novel chemotherapeutic treatment for osteosarcoma.

One of the most common malignant bone cancers in humans is osteosarcoma. The survival period of osteosarcoma patients has increased with the use of multidrug chemotherapy and forceful surgical methods. However, it is still difficult to diagnose patients with growth diseases [28]. Early progression, obstruction with chemotherapy, and modified expression of tumor proteins further promotes osteosarcoma [29]. Hence, more efficient agents are required for a cure. Herein, we established that Leu^{5}AMD hinders the proliferation of Saos-2 osteosarcoma cells and induces apoptosis. Higher concentrations of Leu^{5}AMD (1.2 μM and 6 μM) exhibited significant effects. Additionally, our results indicate that Leu^{5}AMD potently induces apoptosis in Saos-2 cells via cell cycle arrest.

The induction of cell death can be determined from apoptosis using cytostatic drugs [30,31]. These drugs activate different mechanistic pathways, resulting in apoptosis that involves the kindling of ligand units, DNA damage, mitochondrial changes, and so on. We demonstrated that Leu^{5}AMD induces cell cycle arrest upon inhibiting the protein expression of cyclins, indicating that cyclins are effective oncogenes. The inhibitory effects of Leu^{5}AMD on cyclins are consistent with the Leu^{5}AMD-induced apoptosis in Saos-2 osteosarcoma cells.

Acknowledgments: The authors would like to thank the National Natural Science Foundation of China (No.:81301588) for the financial support to carry out this study.

Authors' contribution: Xiaofeng Wu and Zihui Zhou designed the experiments. Xiaofeng Wu did all experiments and wrote the manuscript. Zihui Zhou revised the manuscript. Both the authors approved the final version of the manuscript.

Conflict of interest disclosure: None to declare.

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