Efficacy of celastrol combined with cisplatin in enhancing the apoptosis of U-2OS osteosarcoma cells via the mitochondrial and endoplasmic reticulum pathways of apoptosis

QIANG WANG[1*], XIAOLONG YU[2*], FAN LI[2], XIN LV[2], XIAOXING FU[2], HOUYUN GU[2], HUCHENG LIU[3], JUN LIU[2], MIN DAI[2*] and BIN ZHANG[4*]

[1]Department of Orthopedics, Fujian Longyan First Hospital, Longyan First Affiliated Hospital of Fujian Medical University, Longyan, Fujian 364000; [2]Department of Orthopedics; [3]Multidisciplinary Therapy Center of Musculoskeletal Tumors, The First Affiliated Hospital of Nanchang University; [4]Artificial Joint Engineering and Technology Research Center of Jiangxi Province, Nanchang, Jiangxi 330006, P.R. China

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Abstract. Osteosarcoma is a common primary malignant tumor of bone, and the poor prognosis and low 5-year survival rate have not improved for three decades. The present study aimed to study the effect a combination of celastrol and cisplatin on the human osteosarcoma cell line U-2OS, and to investigate the mechanism by which celastrol/cisplatin induces the apoptosis of osteosarcoma cells. MTT and Annexin V-FITC/PI assays were used to evaluate the effects of combined celastrol/cisplatin on growth and apoptosis, respectively, in U-2OS cells. Morphological changes accompanying cell growth inhibition were observed using a fluorescence microscope. Combination index (CI) analysis was used to evaluate the combinatorial effects of celastrol/cisplatin treatment. Western blotting was used to quantify the expression of apoptosis-associated proteins. It was identified that celastrol/cisplatin inhibited the growth of U-2OS cells in a dose-dependent manner. CI analysis revealed that combined celastrol/cisplatin demonstrated a synergistic effect in U-2OS cells, with CIs ranging from 0.80 to 0.97 at effect levels from IC_{10} to IC_{70}. In addition, it was observed that celastrol/cisplatin upregulated the expression of Bcl-associated X protein, cytochrome *c*, caspase-3 and C/EBP homologous protein, and downregulated the expression of Bcl-2, poly(ADP-ribose) polymerase, 78 kDa glucose-regulated protein and caspase-9, whereas the expression of caspase-8 remained unchanged. To conclude, celastrol/cisplatin induced apoptosis in U-2OS cells via the mitochondrial and endoplasmic reticulum pathways, particularly in the former. Celastrol/cisplatin therefore exhibits potential as a novel therapeutic combination for the treatment of osteosarcoma.

Correspondence to: Professor Min Dai, Department of Orthopedics, The First Affiliated Hospital of Nanchang University, 17 Yong Wai Zheng Street, Nanchang, Jiangxi 330006, P.R. China
E-mail: 214458781@qq.com

Professor Bin Zhang, Artificial Joint Engineering and Technology Research Center of Jiangxi Province, 17 Yong Wai Zheng Street, Nanchang, Jiangxi 330006, P.R. China
E-mail: 1634488248@qq.com

*Contributed equally

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Introduction

Osteosarcoma is a common type of primary malignant tumor of bone that predominantly occurs in children and adolescents, with a high propensity for local invasion and early systemic metastasis (1). It occurs most frequently in the long bones while they are growing, including the humerus, femur and tibia (2). At present, treatment typically includes neoadjuvant chemotherapy, surgical resection and a successive course of chemotherapy following surgery (2). Although the 5-year survival rate for patients with localized osteosarcoma is 60% (3-5), the rate has remained relatively unchanged during the previous three decades. The prognosis is particularly poor for patients with metastasis or relapse, with survival rates of 20-30% (6-8).

The gold standard for osteosarcoma chemotherapy is based on 5 drugs: High-dose methotrexate with leucovorin rescue, doxorubicin, cisplatin, ifosfamide and etoposide (9). However, high-dose chemotherapeutic drugs are not always effective and may cause severe side effects; in addition, multidrug resistant cases are common, particularly to cisplatin, doxorubicin and the majority of neoadjuvant chemotherapy drugs (4,5). Therefore, it is critical to develop novel therapies for the management of osteosarcoma.

Celastrol is a triterpenoid isolated from the 'Thunder of God Vine', which is used in traditional Chinese medicine. Previous studies have demonstrated that it may inhibit growth and encourage apoptosis in a variety of human cancer cell lines, including hepatoma, myeloma, and breast, pancreatic

and gastric cancer cell lines (10-14). Celastrol may also induce the apoptosis of human osteosarcoma cells, via the mitochondrial pathway of apoptosis (15). Therefore, as tumor cells exhibit increasing resistance to traditional chemotherapy drugs including cisplatin, combinatorial treatments, including a combination of cisplatin with celastrol, may assist in improving the efficacy of chemotherapy.

In the present study, the effects of cisplatin, celastrol and combined cisplatin/celastrol on the human osteosarcoma U-2OS cell line was investigated, and the molecular mechanism by which celastrol/cisplatin induce the apoptosis of osteosarcoma cells was examined, with the aim of providing a theoretical basis for their clinical combination.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), PBS, dimethyl sulfoxide (DMSO) and MTT were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was purchased from Beijing Transgen Biotech Co., Ltd. (Beijing, China) and a Hoechst 33258 staining kit was provided by Nanjing Keygen Biotech Co., Ltd. (Nanjing, China). Antibodies against Bcl-2 (cat no. ab32124), Bcl-2-associated X protein (Bax; cat no. ab32503), caspase-3 (cat no. ab13847), caspase-8 (cat no. ab108333), caspase-9 (cat no. ab32539), poly(ADP-ribose) polymerase (PARP; cat no. ab219953), cytochrome *c* (cat no. ab133504), 78 kDa glucose-regulated protein (GRP78; cat no. ab21685) and C/EBP-homologous protein (CHOP; cat no. ab11419) were purchased from Abcam (Cambridge, MA, USA). β-actin (cat no. 8H10D10) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse antibody; cat no. 14709S; and anti-rabbit antibody; cat no. ZB-2306) were purchased from Cell Signaling Technology, Inc. and Beijing Transgen Biotech Co., Ltd., respectively. An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was provided by Nanjing Keygen Biotech Co., Ltd.

Celastrol and cisplatin were obtained from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). Stock solutions of celastrol were prepared by dissolving the celastrol powder in DMSO to a concentration of 20 M, and stock solutions of cisplatin were prepared by dissolving the cisplatin powder in saline to 1 mg/l; these were stored at -20˚C. Working solutions of celastrol and cisplatin were prepared by diluting the stock solution with culture medium. The final concentration of DMSO in the medium was <0.1%.

Cell culture. Cells of the human osteosarcoma U-2OS cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 μ/ml penicillin, and 100 μg/ml streptomycin. Cells were kept in a humidified atmosphere containing 5% CO_2 at 37˚C. Cells used in the present study had been subjected to <20 cell passages and were in the logarithmic growth phase.

Quantification of cell viability by MTT assay. Cells were cultured in 96-well plates at a concentration of $1x10^4$ cells/well and cell viability was determined using an MTT colorimetric assay. Cells were treated with various concentrations of celastrol (1, 2, 3, 4 and 5 μM), cisplatin (2, 4, 6, 8 and 10 μg/ml), or a combination of celastrol/cisplatin at each final concentration, for 24, 36 or 48 h; control cells were treated with 0.02% DMSO. Following the incubation period, 20 μl of MTT (5 mg/ml in PBS) was added and the plates were incubated at 37˚C for an additional 4 h. The formazan precipitate was then dissolved in 150 μl DMSO and agitated for 10 min. Absorbance was measured at 490 nm using a universal microplate reader (ELISA Reader Model EXl800; BioTek Instruments, Inc., Winooski, VT, USA). Cell growth was expressed as the relative percentage of viability by comparing the absorbance of treated vs. control cells. Each experiment was repeated 3 times at each time point/dose.

Quantification of apoptosis by Annexin V-FITC/PI staining assay. To assess the induction of apoptosis by celastrol and cisplatin, U-2OS cells were stained with the Annexin V-FITC/PI kit. U-2OS cells were seeded in 6-well culture plates ($1.5x10^5$ cells/well) and incubated for 24 h; the cells were incubated with celastrol (2.6 μM) and/or cisplatin (6.1 mg/l) for 48 h and collected by trypsinization, without EDTA. Following two rounds of washing with PBS at 4˚C, the cell pellets were re-suspended in 400 μl ice-cold 1X binding buffer at a density of ~$1x10^6$ cells/ml and incubated in Annexin V-FITC and PI (10 μg/ml) at room temperature for 10 min in the dark. Samples were analyzed using a flow cytometer within 1 h of staining. BD Accuri C6 Software 1.0.264.21 (BD Biosciences, Franklin Lakes, NJ, USA) was used for analysis, and the experiment was repeated 3 times.

Hoechst 33258 staining of U-2OS cells. Cells were incubated for 48 h with the IC_{50} of celastrol, cisplatin or celastrol combined with cisplatin, harvested, fixed with 4% paraformaldehyde for 30 min at 25˚C, washed 3 times with ice-cold PBS and stained with 10 mg/l Hoechst 33258 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 10 min in the dark at room temperature. The stained nuclei were observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan; magnification, x100) with excitation at 350 nm and emission at 460 nm. This experiment was repeated 3 times.

Western blot analysis. U-2OS cells were cultured in 6-well plates ($2x10^5$ cells/well). Following treatment with the indicated concentration of celastrol and/or cisplatin [group 1, celastrol (2.6 μM); group 2, cisplatin (6.1 mg/l); group 3, celastrol (2.6 μM) combined with cisplatin (6.1 mg/l)] for 48 h and, subsequent to washing with ice-cold PBS, the cells were collected and lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The homogenates were centrifuged at 24,148.8 x g for 10 min at 4˚C and the supernatant fraction was collected for immunoblotting. Protein concentrations were determined with a BCA assay using bovine serum albumin as the standard. Equal quantities (45 μg) of protein were loaded and separated by electrophoresis on 12% SDS-PAGE gels under reducing conditions at 110 V for 2 h. Following electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes in a Tris-glycine transfer buffer (3.05 g Tris, 14.4 g glycine and 200 ml methanol solute in 1,000 ml water) using a semi-dry blotting system, and

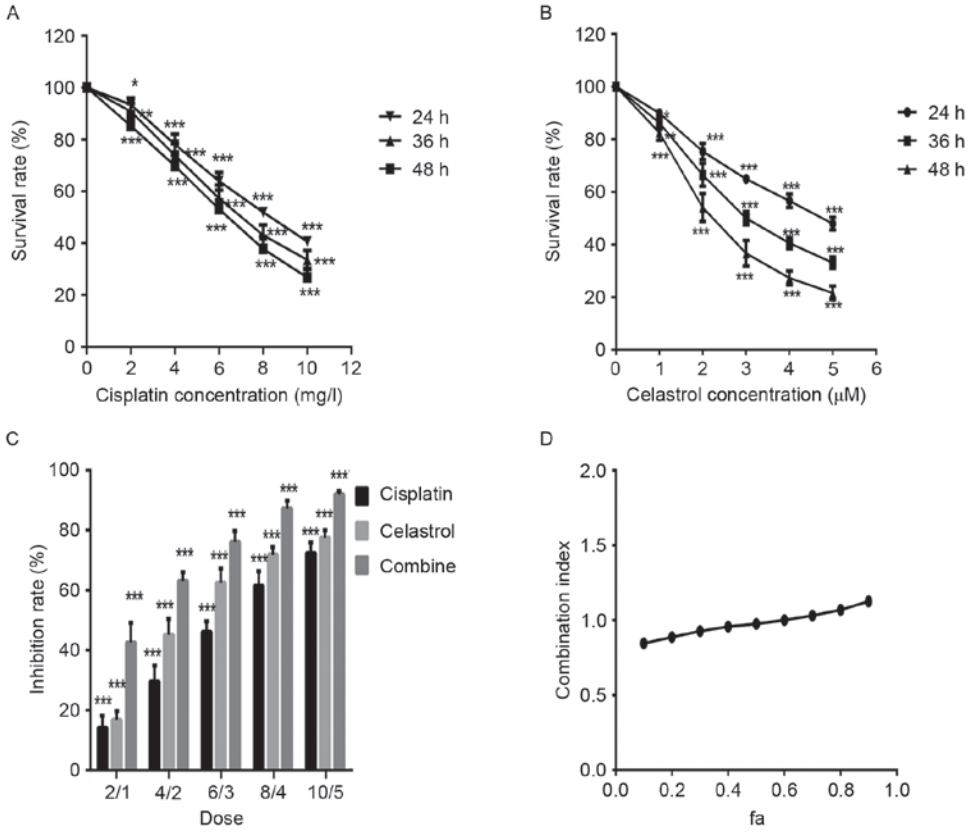

Figure 1. Effect of cisplatin, celastrol or a combination on the viability of U-2OS cells. (A) Dose- and time-effect of cisplatin on the viability of U-2OS cells. (B) Dose- and time-effect of celastrol on the viability of U-2OS cells. (C) The viability of U-2OS cells following treatment with celastrol and cisplatin at each concentration for 48 h. (D) The combination index. Data are expressed as means ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. control (0 μM). fa, fraction affected.

incubated with antibodies against Bcl-2, Bax, cytochrome *c*, caspase-3, -8 and -9, GRP78, CHOP, PARP and β-actin (dilution, 1:1,000) overnight at 4˚C. The PVDF membranes were washed in Tris-buffered saline with Tween-20 (TBST) 3 times and secondary HRP-conjugated antibodies (dilution, 1:2,000) were added for 2 h at room temperature. The PVDF membranes were then re-washed in TBST 3 times. Bound antibodies were detected using the ECL Plus Western Blotting Detection system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and a LAS-1000 Plus Image Analyzer (Fujifilm Holdings Corporation, Tokyo, Japan). The western blots were quantified by densitometric analysis using Image Lab 4.0.153407 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). This experiment was repeated 3 times.

Combination index (CI) analyses. The multiple drug effect analysis developed by Chou and Talalay (16), which is based on a median-effect principle, was used to calculate combined drug effects; results are presented as a CI. Synergism, additivity and antagonism are indicated by CI<1, CI=1, and CI>1, respectively.

Statistical analysis. Data were analyzed using SPSS 17 (SPSS, Inc., Chicago, IL, USA). Quantitative data are expressed as the mean ± standard deviation. Statistical analysis was performed using a paired Student's t-test and one-way analysis of variance. Fisher's Least Significant Difference test was used as a post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Celastrol acts synergistically with cisplatin to inhibit the growth of U-2OS cells. The effect of celastrol and cisplatin on the viability of U-2OS cells was determined by an MTT assay. U-2OS cells were treated with various concentrations of celastrol and cisplatin for 24, 36, and 48 h. As demonstrated in Fig. 1A and B, celastrol and cisplatin inhibited the growth of U-2OS cells in a dose-dependent manner. The IC_{50} value for U-2OS cells was 2.6 μM at 48 h for celastrol and 6.1 mg/l for cisplatin.

The effect of a combination of celastrol and cisplatin on the growth of U-2OS cells was also examined, to investigate potential synergistic effects. Combined treatment with celastrol/cisplatin markedly decreased U-2OS cell growth, compared with celastrol or cisplatin alone (Fig. 1C). CI values, based on the Chou-Talalay equation (12), were calculated to estimate the efficacy of celastrol/cisplatin. The CI-'fraction affected' curve suggested that combined treatment with celastrol/cisplatin exhibited a synergistic effect on cell growth inhibition, with CI values ranging from 0.80 to 0.97 at effect levels from IC_{10} to IC_{70} (Fig. 1D).

Celastrol acts synergistically with cisplatin to induce the apoptosis of U-2OS cells. Annexin V-FITC/PI double staining-based FACS analysis was used to investigate the effect of celastrol/cisplatin on apoptosis of U-2OS cells. The apoptosis rate was 7.9±1.4% in the control group, 30.2±2.3%

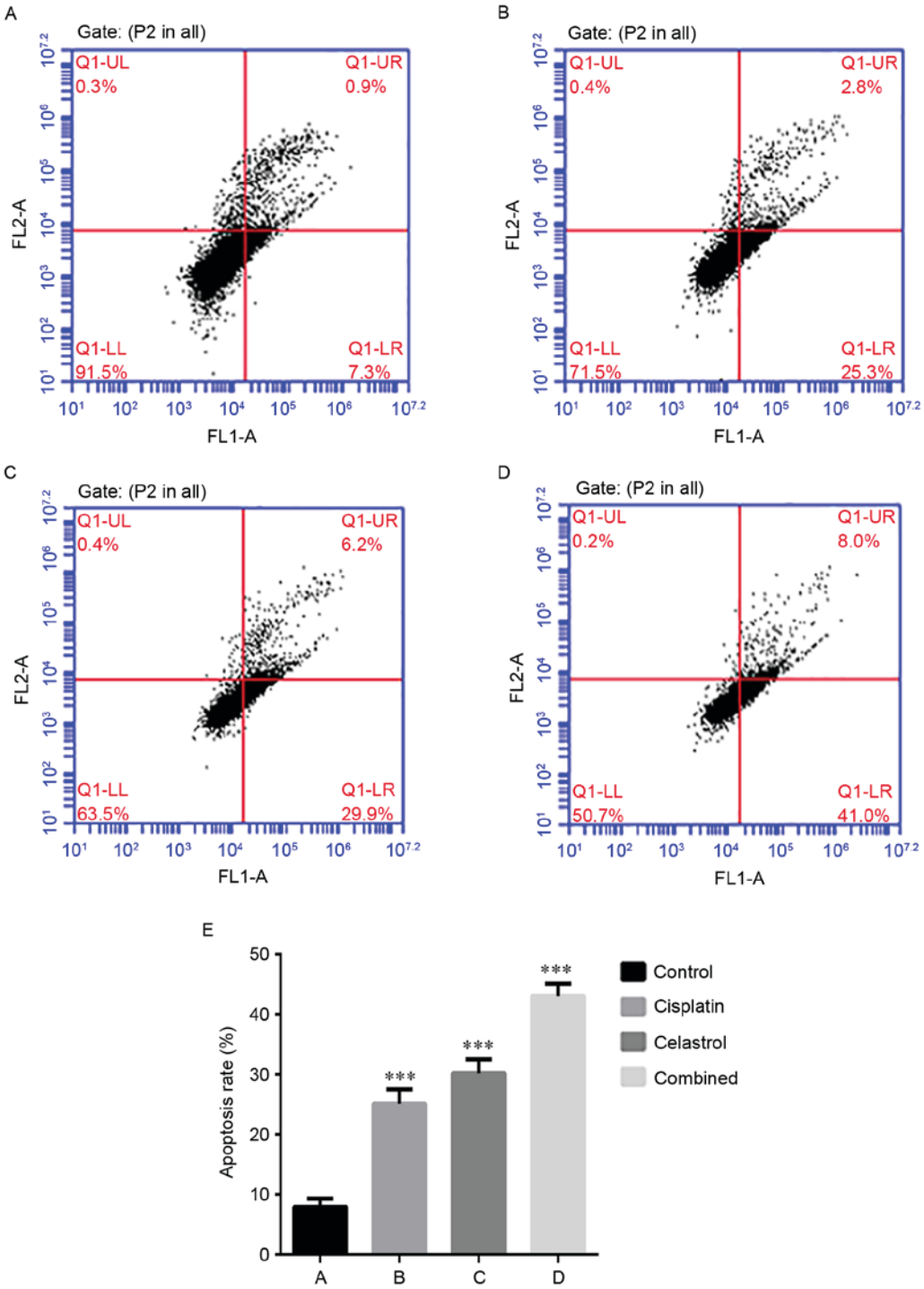

Figure 2. Annexin V-FITC/PI staining assay. Representative graphs obtained by flow cytometric analysis following double-staining with Annexin V-FITC/PI. Compared with (A) the control cells (0 μM, 7.9±1.25%), the percentage of apoptotic U-2OS cells was increased to 28.5±0.9, 35.4±1.4 and 48.5±1.6% following treatment with (B) celastrol, (C) cisplatin and (D) celastrol combined with cisplatin for 48 h, respectively. (E) Quantification of parts A-D. Data are expressed as means ± standard deviation. ***P<0.001 vs. control (0 μM). FITC, fluorescein isothiocyanate; PI, propidium iodide.

in cells treated with celastrol (2.6 μmol/l), 25.1±2.4% in cells treated with cisplatin (6.1 mg/l) and 43.0±2.1% in cells treated with celastrol/cisplatin (Fig. 2). This result suggests that celastrol/cisplatin in combination exhibit an enhanced ability to induce apoptosis. In addition, cell morphology analysis performed subsequent to the staining of U-2OS cells with the fluorescent DNA-binding dye Hoechst 33258 supported the observation that the number of apoptotic cells was higher following celastrol/cisplatin treatment compared with either treatment alone; no morphological signs of apoptosis were observed in untreated cells, in contrast to the treated cells (Fig. 3).

Effect of celastrol/cisplatin on the expression of caspase-family proteins. The caspase cascade reaction is critical in the process of apoptosis; therefore, the expression levels of caspase-3, -8 and -9 were assessed. Caspase-3 cleavage was observed, and the expression level of caspase-9

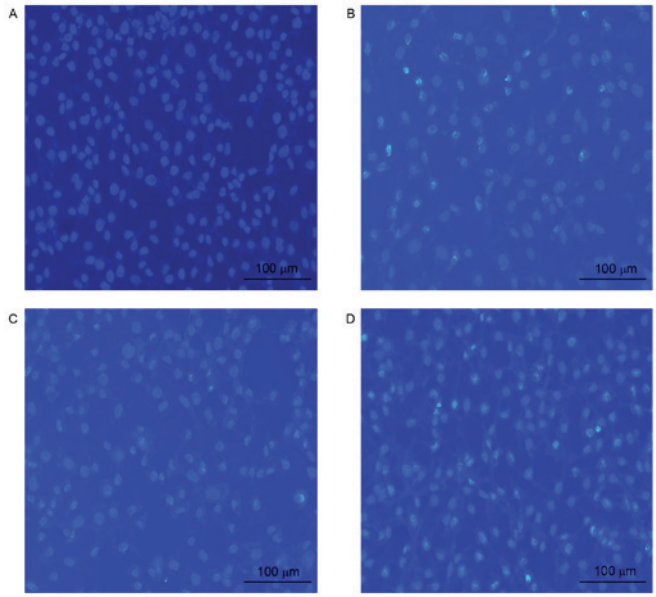

Figure 3. Hoechst 33258 staining of U-2OS cells. Apoptotic nuclei exhibited condensed or fragmented DNA brightly stained by Hoechst 33258 following 48 h of staining. Representative images of (A) the control, (B) celastrol-treated cells (C) cisplatin-treated cells and (D) combination treatment. Magnification, x200.

was downregulated in the treatment groups, particularly following celastrol/cisplatin treatment. However, the expression levels of caspase-8 remained unchanged in cells treated with celastrol/cisplatin. The cleavage of PARP, a key cellular substrate of caspases, was observed; the relative expression of intact PARP was significantly reduced by all treatments, particularly the combination treatment. The results suggest that apoptosis induced by celastrol/cisplatin was associated with the caspase cascade ($P<0.001$; Figs. 4 and 5).

Effects of celastrol/cisplatin on the expression of proteins associated with mitochondrial apoptosis, Bcl-2, Bax, and cytochrome c. To investigate the molecular mechanism by which celastrol/cisplatin induces apoptosis in U-2OS cells, the expression levels of proteins associated with the mitochondrial pathway of apoptosis, including Bcl-2, Bax, and cytochrome *c*, were assessed. Western blot analysis revealed that following celastrol/cisplatin treatment, the expression levels of cytochrome *c* and Bax were upregulated, but that the expression level of Bcl-2 was downregulated, compared with either treatment alone or the control (Figs. 5 and 6; $P<0.001$). This confirmed that celastrol/cisplatin may regulate the expression of the Bcl-2 family proteins to activate the mitochondrial apoptotic pathway in U-2OS cells.

Effects of celastrol/cisplatin on the expression of proteins associated with the endoplasmic reticulum pathway of apoptosis. It was previously demonstrated that cisplatin treatment may induce endoplasmic reticulum stress (17); therefore, we hypothesized that celastrol/cisplatin activates the endoplasmic reticulum pathway of apoptosis in U-2OS cells. Consequently, the expression levels of the endoplasmic reticulum stress-associated protein GRP78 and the endoplasmic reticulum stress-apoptosis-associated protein CHOP were assessed by western blot analysis. The expression level of GRP78 was downregulated, whereas CHOP was upregulated following celastrol/cisplatin treatment, compared with either treatment alone or the control ($P<0.001$; Fig. 7). These data demonstrated that celastrol/cisplatin treatment may induce endoplasmic reticulum stress and induce the progress of apoptosis via the endoplasmic reticulum apoptotic pathway.

Discussion

Apoptosis, genetically controlled cell suicide, is an important mechanism by which numerous anticancer drugs elicit their effects. Apoptosis is an innate mammalian cellular response for eliminating abnormal or redundant cells. The two classical pathways for the induction of apoptosis are the cell death receptor pathway and the mitochondrial pathway (18-21); in addition, several studies have identified an additional pathway, the endoplasmic reticulum pathway (22,23).

In the mitochondrial pathway, downstream caspase activation is regulated by members of the Bcl-2 family (24-26). The apoptosis-associated mitochondrial outer membrane permeabilization requires pro-apoptotic Bax-like proteins, which regulate mitochondrial pore formation; the anti-apoptotic Bcl-2-like proteins are functionally distinct in their role in apoptosis. The ratio of Bax to Bcl-2 is important for determining the release of apoptogenic proteins from the mitochondrial intermembrane space, including cytochrome *c*, which activates caspase-9 (24-26). Firstly, caspase-9 is activated, following which it activates the downstream caspase-3, which causes the cleavage or degradation of various key cellular substrates, including PARP, leading to apoptosis (24-26). In the cell death receptor pathway, also termed the extrinsic pathway, the interaction between the Fas cell surface receptor and the Fas ligand promotes caspase-8 activation (27,28). Therefore, the present study quantified the expression of Bcl-2, Bax, caspase-3, -8, -9 and PARP in U-2OS cells (29,30).

The data of the present study indicated that celastrol/cisplatin-induced apoptosis was affected by the alteration of the Bax/Bcl-2 ratio and activation of caspase-3 and -9, but not caspase-8. Cleavage of PARP was also observed. These results suggested that celastrol/cisplatin-induced apoptosis is induced via the mitochondrial pathway in U-2OS cells.

In the endoplasm reticulum pathway, apoptosis is induced by endoplasmic reticulum stress (22,23). The endoplasmic reticulum stress-associated protein GRP78 and the endoplasmic reticulum stress apoptosis-associated protein CHOP are transcription factors specific to the process of endoplasmic reticulum stress (31,32). A previous study revealed that CHOP upregulated the expression of pro-apoptotic Bax-like proteins and downregulated anti-apoptotic members, including Bcl-2 (33-35). In the present study, western blot analysis indicated that levels of GRP78 were downregulated and those of CHOP were upregulated, accompanied by an increase in the Bax/Bcl-2 ratio, following celastrol/cisplatin treatment. These data indicate that celastrol/cisplatin-induced apoptosis is triggered via the endoplasm reticulum pathway in U-2OS cells.

The present study demonstrated that celastrol acts synergistically with cisplatin to inhibit the growth of U-2OS cells through the induction of apoptosis. CI analyses revealed that celastrol/cisplatin exhibit synergy in U-2OS cells, with

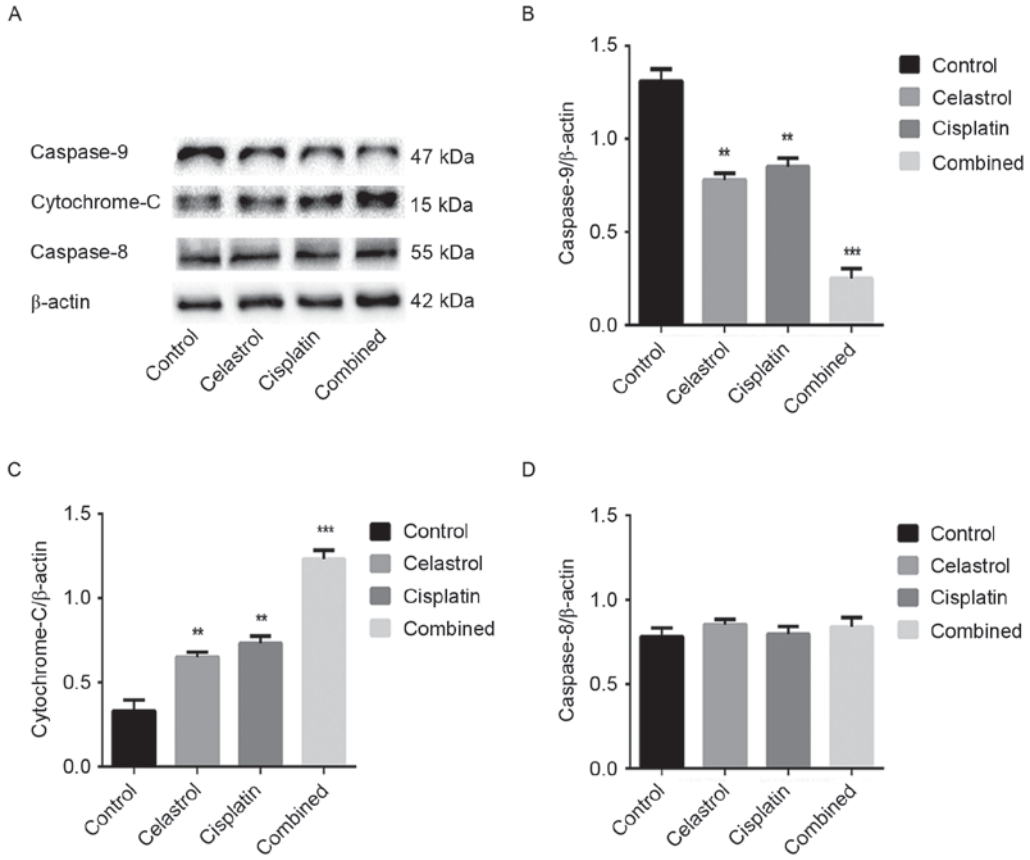

Figure 4. Effect of celastrol and cisplatin on the protein expression levels of caspase-8, 9 and cytochrome *c* in U-2OS cells. (A) The protein expression levels of caspase-9, caspase-8 and cytochrome *c* were analyzed by western blot analysis. β-actin was used as the internal control for the western blot analysis. The quantified relative protein expression levels of (B) Caspase-9, (C) cytochrome *c* and (D) caspase-8 in celastrol/cisplatin-treated and control cells. The data are expressed as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. control cells.

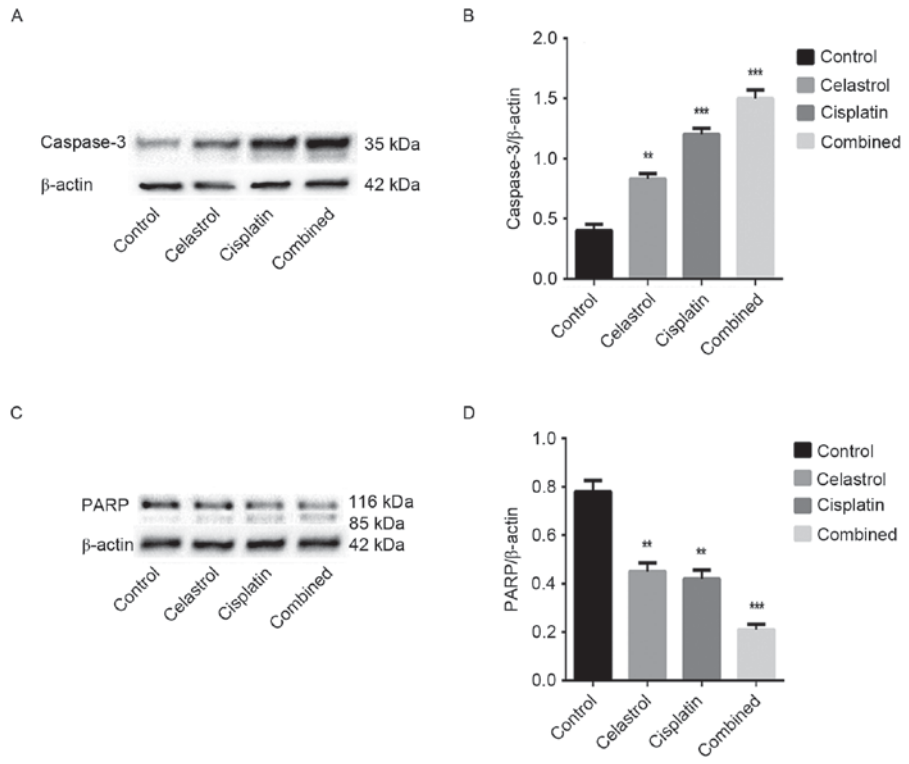

Figure 5. Effect of celastrol and cisplatin on the protein expression levels of caspase-3 and PARP in U-2OS cells. (A) Representative blotting image of caspase-3; (B) quantification of relative caspase levels. (C) Representative blotting image of PARP; (D) quantification of relative PARP levels. β-actin was used as the internal control. The data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. control cells. PARP, poly(ADP-ribose) polymerase.

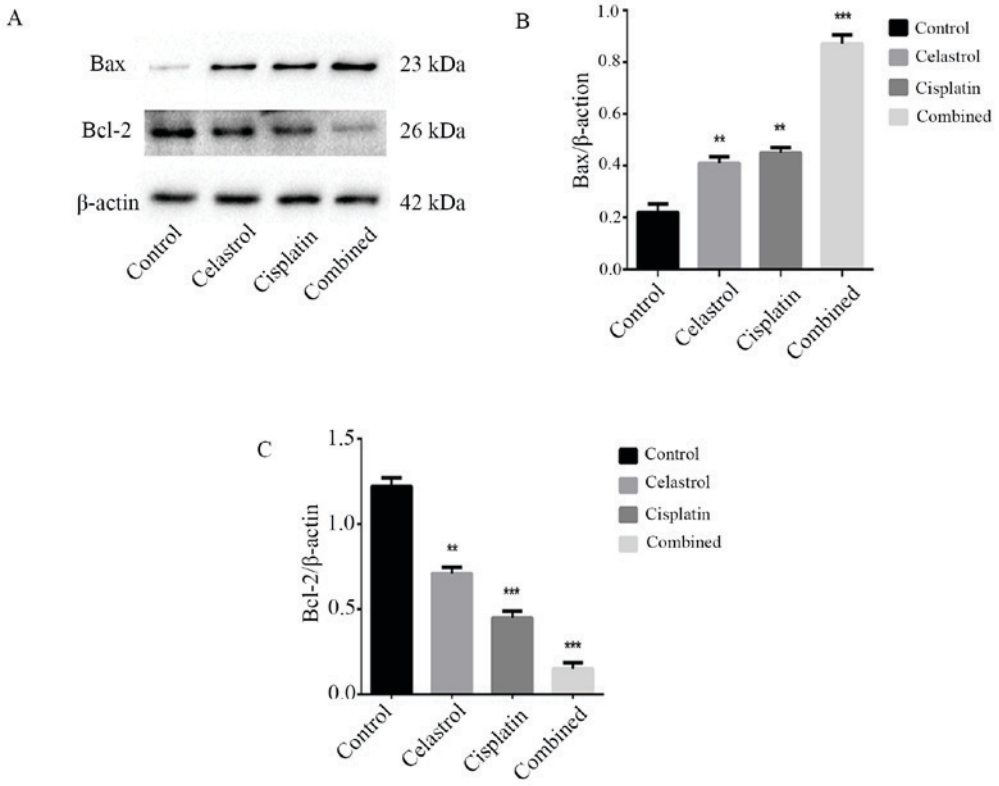

Figure 6. Effect of celastrol and cisplatin on the protein expression levels of Bcl-2 and Bax in U-2OS cells. (A) The protein expression levels of Bax and Bcl-2 were analyzed by western blot analysis. β-actin was used as the internal control for the western blot analysis. Quantification of the relative protein expression of (B) Bax and (C) Bcl-2 in celastrol/cisplatin-treated and control cells. The data expressed are the mean ± standard deviation. **P<0.01, ***P<0.001 vs. control cells. Bax, Bcl-associated X protein.

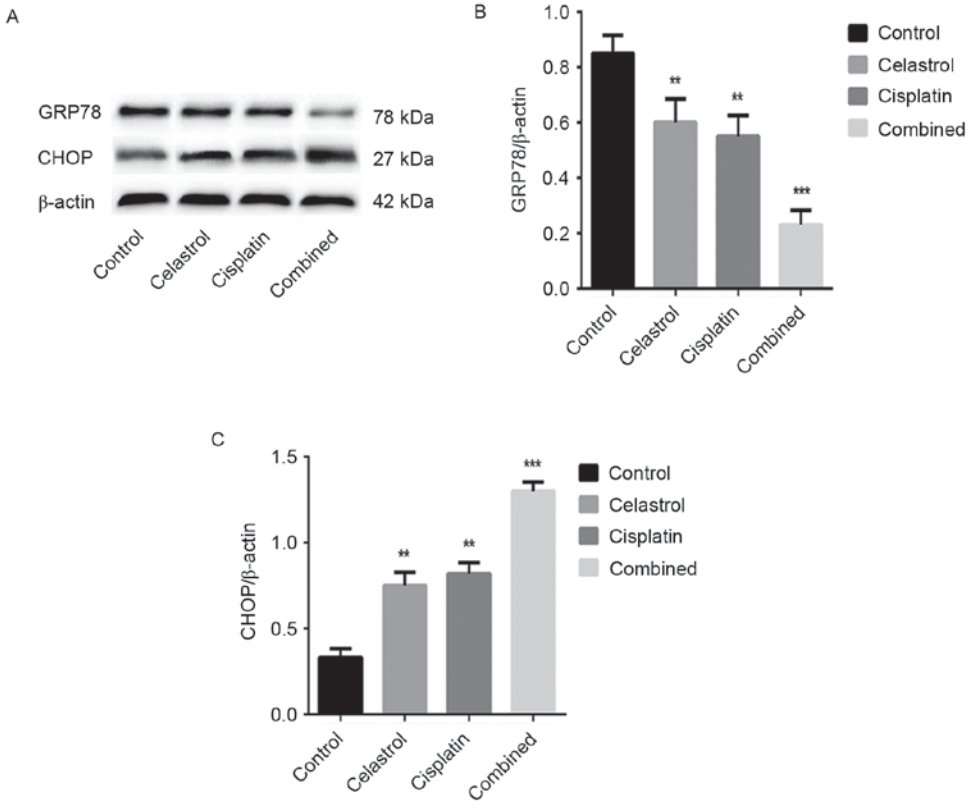

Figure 7. Effect of celastrol and cisplatin on the protein expression levels of GRP78 and CHOP in U-2OS cells. (A) The protein expression levels of GRP78 and CHOP were analyzed by western blot analysis. β-actin was used as the internal control for the western blot analysis. Quantification of the relative protein expression levels of (B) GRP78 and (C) CHOP in celastrol/cisplatin-treated and control cells. The data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. control cells. GRP78, 78 kDa glucose-regulated protein; CHOP, C/EBP homologous protein.

CIs ranging from 0.80 to 0.97 at effect levels ranging from IC_{10} to IC_{70}. Celastrol/cisplatin-induced apoptosis was triggered via the mitochondrial and endoplasmic reticulum pathways in U-2OS cells, particularly the former. Therefore, celastrol/cisplatin exhibits potential as a novel therapeutic agent for the treatment of osteosarcoma. Additional studies are required to improve the understanding of the synergistic effects of these drugs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MD, BZ and HL contributed to the experimental design. QW, XY and FL contributed to experiments and manuscript writing. XL, XF, HG and JL contributed to data analysis and interpretation. All authors approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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