

# Quercetin induces apoptosis in the methotrexate-resistant osteosarcoma cell line U2-OS/MTX300 via mitochondrial dysfunction and dephosphorylation of Akt

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**Abstract.** Quercetin is the most abundant polyphenolic flavonoid found in plants. Several studies suggest that it has potent anticancer effects. The present study examines the apoptosis-inducing activity and the underlying mechanism of action of quercetin in a methotrexate (MTX)-resistant osteosarcoma model. Our results showed that quercetin inhibited cell viability in a dose-dependent manner and there was no cross-resistance between MTX and quercetin in U2-OS/MTX300 cells. The induction of apoptosis was observed by flow cytometry and fluorescence staining experiments. Quercetin-induced apoptosis was accompanied by a significant reduction of mitochondrial membrane potential, release of mitochondrial cytochrome c to the cytosol, activation of caspase-3, down-regulation of Bcl-2, p-Bad and up-regulation of Bax. A remarkable dephosphorylation of Akt was also detected after quercetin treatment. Furthermore, transduction with constitutively active Akt protected against the quercetin-induced dephosphorylation of Akt and Bad as well as poly(ADP-ribose)polymerase (PARP) degradation, while combined treatment with quercetin and LY294002 enhanced the dephosphorylation of Akt, Bad and PARP cleavage in U2-OS/MTX300 cells. Taken together, our results demonstrate that quercetin-induced apoptosis in the MTX-resistant osteosarcoma cells U2-OS/MTX300 was mediated via mitochondrial dysfunction and dephosphorylation of Akt.

## Introduction

Osteosarcoma is the most common primary malignant bone tumor diagnosed in children and adolescents (1). Due to the

development of adjuvant and neo-adjuvant chemotherapy, local control of osteosarcoma and overall survival have improved significantly. Currently used chemotherapy regimens are based on a combination of methotrexate (MTX), doxorubicin, cisplatin and ifosfamide. MTX is the most active one (2). Despite improvements of chemotherapy, a considerable number of osteosarcoma patients develop MTX resistance and die as a result of disease progression (3). Additional therapeutic agents should be evaluated to improve the survival of MTX-resistant osteosarcoma patients.

Quercetin is the most abundant molecule in the extensive class of polyphenolic flavonoids and is found ubiquitously in plants and foods (4). The average daily dietary intake of quercetin is estimated to be 16 mg (5). The bioactivities of quercetin are complex and include antioxidative, antiviral, antibacterial and anti-inflammatory effects (4,6,7). Recently, quercetin was found to possess strong anticancer properties in colon cancer (8-11), breast cancer (12,13), leukemia (14-16), hepatocellular carcinoma (17), pancreatic carcinoma (18), salivary adenoid cystic carcinoma (19) and lung cancer (20). Although the anti-tumor effects of quercetin have been examined in several tumors, very little is known about its effects in osteosarcoma cells. Thus, in this study we used a MTX-resistant model to determine the effects of quercetin on the MTX-resistant osteosarcoma cells, and elucidate the underlying mechanism.

## Materials and methods

**Drugs and reagents.** Quercetin, MTT (methyl thiazolyl tetrazolium), Hoechst 33258, LY294002, and N-benzyloxy-carbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-fmk) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies for cytochrome c, Bax, Bcl-2, phospho-Bad (Ser136), Bad, phospho-AKT (Ser473), Akt and  $\beta$ -actin were obtained from Cell Signaling (Danvers, MA, USA), while the antibodies for PARP and cleaved PARP were purchased from Abcam (Cambridge, MA, USA).

**Cell lines and culture.** The MTX-sensitive osteosarcoma cell line U2-OS and the MTX-resistant variant U2-OS/MTX300

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were kindly provided by Dr M. Serra (Istituti Ortopedici Rizzoli, Bologna, Italy). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA), supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), penicillin (10000 U/l) and streptomycin (100 mg/l) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The MTX300-resistant variant was continuously cultured in the medium with 300 µg/l MTX (21). The murine amphotropic retroviral packaging cell line, PA317, was obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences and maintained in DMEM medium containing 10% fetal calf serum and antibiotics.

**MTT assay.** After quercetin treatment for 48 h, 50 µl of 1 mg/ml MTT solution was added to each well of the plate. DMSO (100 µl) was added to each well 4 h later for optical reading at 570 nm. The concentrations required to inhibit cell growth by 50% (IC<sub>50</sub>) were calculated from the cytotoxicity curves (Bliss's software; Bliss Co., CA, USA).

**Clonogenic assay.** U2-OS/MTX300 were treated with varying concentrations of quercetin for 24 h. Two hundred viable cells per well were replated, then cultured in regular medium for 10 days and stained with 0.5% crystal violet solution for 30 min. The staining solution was decanted and the cells were washed with deionized H<sub>2</sub>O. Individual colonies were counted.

**Fluorescence staining.** Cells were collected by centrifugation (1,500 x g, 5 min) following 48 h quercetin treatment, washed twice with phosphate-buffered saline (PBS), and stained with Hoechst 33258 (10 ml, 10 mg/l) for 10 min at 37°C to visualize nuclei. An Olympus photomicroscope (Olympus, Tokyo, Japan) was used to observe the treatment-induced morphological changes.

**Annexin V/PI staining assay.** Cells were treated with 0, 10, 25 or 50 µM quercetin for 48 h. Next, the cells were collected, washed twice with PBS and resuspended to a concentration of 1x10<sup>6</sup> cells/ml and incubated with Annexin V-FITC and propidium iodide for 30 min in darkness. Cell apoptosis was analyzed by FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were analyzed by the CellQuest software (Becton-Dickson).

**Assay of mitochondrial membrane potential.** The mitochondrial membrane potential was measured by using rhodamine 123. After treatment with/without quercetin for 48 h, cells were rinsed with PBS, rhodamine (10 µM) was loaded, and then the cells were incubated for 10 min at 37°C. Fluorescence intensity of the cells was examined in a Perkin-Elmer L15B fluorescence spectrophotometer (Perkin-Elmer; Waltham, MA, USA) at an excitation and emission wavelength of 480 and 530 nm, respectively. The resultant fluorescence intensity was used as an arbitrary unit representing the mitochondrial transmembrane potential.

**Detection of mitochondrial cytochrome c release.** After treatment, the cells were harvested and resuspended in ice-cold cell lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM

MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, protease inhibitor mixture) and incubated for 1 h at 4°C. The lysates were then homogenized. Homogenates were centrifuged at 12,000 x g for 5 min at 4°C. Supernatants were collected and further centrifuged at 100,000 x g for 30 min at 4°C to obtain the cytosolic fraction, which was then analyzed for cytochrome c by Western blotting.

**Assay of caspase-3 activity.** The caspase-3 activity was measured by using a caspase-3 colorimetric assay kit (caspase colorimetric assay kits; BioVision, Inc.) as directed by the manufacturer. The optical density was measured at 405 nm by a spectrophotometer.

**Western blot analysis.** Cells were lysed with a protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA). For each sample, 30 µg of protein were run on an SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes. Next, the membranes were incubated with primary antibodies for 12 h at 4°C followed by 3 washes in Tris-buffered saline with Tween-20 (TBST) for 5 min each. Secondary antibody conjugated with horseradish peroxidase was added to the membrane and incubated for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence detection system.

**Transduction.** The pLNCX and pLNCX-Myr-Akt retroviral plasmids were purchased from Addgene (Cambridge, MA, USA). PA317 was transfected with pLNCX and pLNCX-Myr-Akt by DOSPER reagents (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. Next, the supernatants were collected 48 h after transfection. U2-OS/MTX300 cells were then incubated with individual retroviral supernatant for 6 h in the presence of 2 µg/ml polybrene (Sigma Chemical Co.) on two successive days. Cells were then incubated at 37°C in a CO<sub>2</sub> incubator for 24 h prior to quercetin treatment.

**Statistical analysis.** All data were derived from at least 3 independent experiments and the results are expressed as the mean ± SD. Differences were assessed using the Student's t-test or one-way ANOVA. P<0.05 was deemed statistically significant. All analyses were performed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

## Results

**Effects of quercetin on the viability of U2-OS/MTX300 cells.** Quercetin demonstrated a potent cytotoxic effect in both the MTX-sensitive U2-OS cells and the MTX-resistant U2-OS/MTX300 cells in a dose-dependent manner (Fig. 1A). The IC<sub>50</sub> values of quercetin for the U2-OS and the U2-OS/MTX300 cells were 24±5 and 22±4 µM, respectively; these differences were not statistically significant (P>0.05). Similarly, quercetin significantly and dose-dependently decreased U2-OS/MTX300 colony formation compared with untreated controls (P<0.05; Fig. 1B and C). These initial results indicate that quercetin inhibits the viability of MTX-resistant osteosarcoma cells *in vitro* and there is no cross-resistance between MTX and quercetin in U2-OS/MTX300 cells.

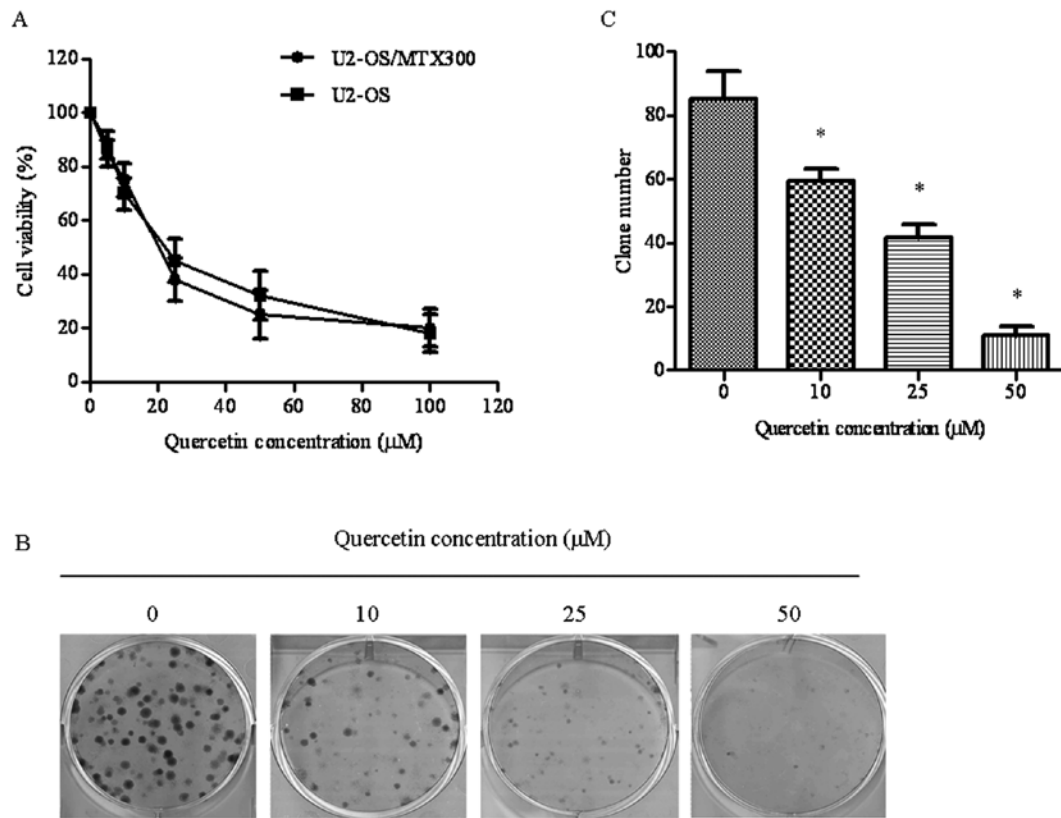


Figure 1. Cytotoxicity of quercetin in U2-OS/MTX300 cells. (A) U2-OS and U2-OS/MTX300 cells were incubated with 0, 5, 10, 25, 50 or 100  $\mu$ M of quercetin for 48 h. The cell survival rate was determined by the MTT assay. The cytotoxicity is expressed as the percentage of cell survival rate compared with the control. The data are expressed as the mean  $\pm$  SD. (B) U2-OS/MTX300 cells were pretreated with 0, 10, 25 or 50  $\mu$ M of quercetin for 24 h, and the effect of quercetin on the colony formation was detected. (C) The number of clones are expressed as the mean  $\pm$  SD. \* $P < 0.05$  vs. control.

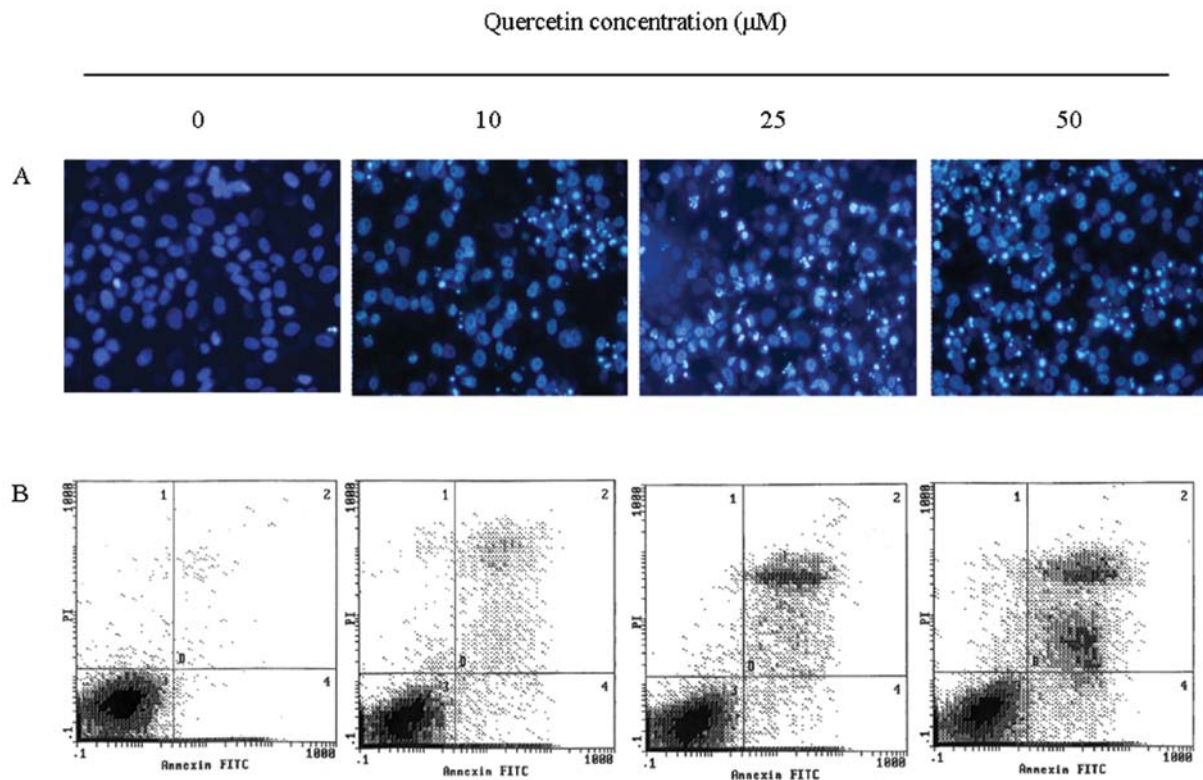


Figure 2. Apoptosis-inducing effects of quercetin in U2-OS/MTX300 cells. (A) Cells were incubated with 0, 10, 25 or 50  $\mu$ M quercetin for 48 h and then Hoechst staining was performed to detect the morphology change. Each picture was taken at a magnification of  $\times 100$ . (B) Annexin V/PI staining assay were used to detect the apoptosis rate.

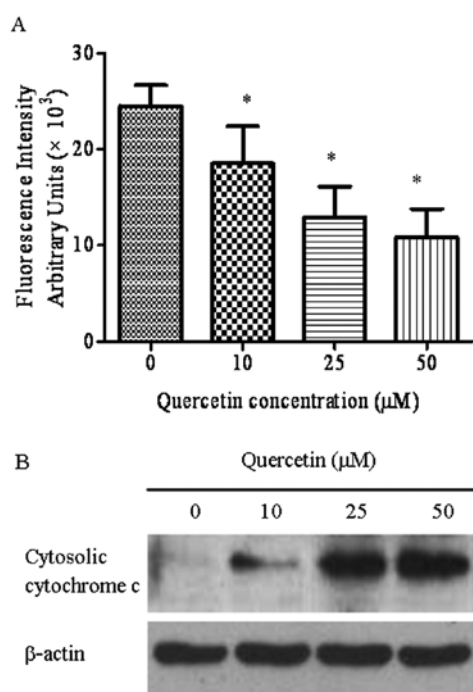


Figure 3. Measurement of mitochondrial membrane potential and cytoplasmic cytochrome c release in U2-OS/MTX300 cells during quercetin treatment. (A) U2-OS/MTX300 cells were incubated without or with quercetin for 48 h. The mitochondrial membrane potential was measured. The data are expressed as mean  $\pm$  SD of 3 determinations, \* $P < 0.05$  vs. the control group. (B) Cytoplasmic cytochrome c release was detected by Western blotting.

*Quercetin induced apoptosis of U2-OS/MTX300 cells.* Fluorescence staining and flow cytometry analysis were used to examine whether quercetin could induce apoptosis in U2-OS/MTX300 cells. As shown in Fig. 2A, quercetin-treated cells presented with several hallmark characteristics of apoptosis, such as chromatin condensation, nuclear fragmentation and the appearance of apoptotic bodies after quercetin treatment. The apoptosis rates in cells treated with 0, 10, 25 or 50  $\mu\text{M}$  quercetin for 48 h were  $3 \pm 2\%$ ,  $13 \pm 4\%$ ,  $22 \pm 2\%$  and  $34 \pm 5\%$ , respectively (Fig. 2B). The P-value was  $< 0.05$  between the control group and all quercetin treatment groups. Collectively, these results suggest that quercetin suppresses cell viability by inducing apoptosis.

*Changes of mitochondrial membrane potential and release of cytochrome c from mitochondria.* To ascertain the role of the mitochondria in quercetin-induced apoptosis in U2-OS/MTX300 cells, the mitochondrial membrane potential as well as cytochrome c levels in the cytosol were examined. Quercetin decreased the mitochondrial membrane potential in a dose-dependent manner (Fig. 3A). Concomitantly, cytochrome c translocation to the cytosol was confirmed by Western blotting (Fig. 3B). These data strongly support the hypothesis that mitochondria dysfunction is involved in quercetin-induced apoptosis in U2-OS/MTX300 cells.

*Caspase-3 was activated after quercetin treatment.* Caspase-3 is a central molecule in the apoptotic cascade due to its ability to

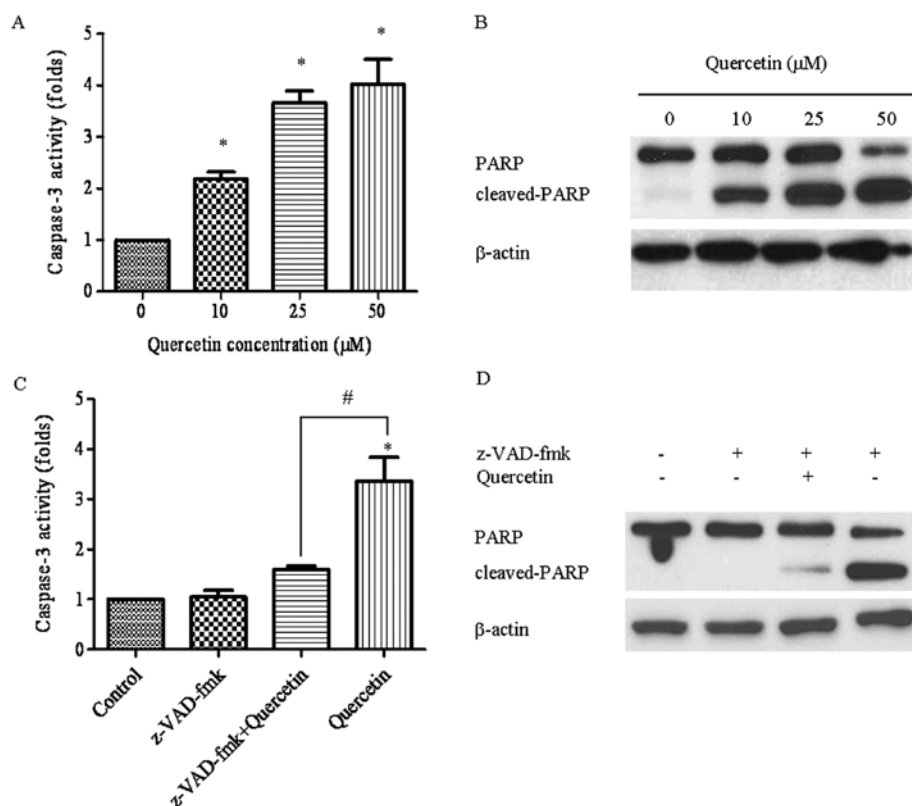


Figure 4. Activation of caspase-3 in U2-OS/MTX300 cells by quercetin. (A) After quercetin treatment, caspase-3 activity was detected by a colorimetric assay. \* $P < 0.05$  vs control (B) The level of PARP and cleaved-PARP were detected by Western blotting. (C) Effect of z-VAD-fmk on the quercetin-induced caspase-3 activation. U2-OS/MTX300 cells were pretreated with/without z-VAD-fmk (25  $\mu\text{M}$ ) for 2 h and then incubated with quercetin (25  $\mu\text{M}$ ) for 48 h. All values are the mean  $\pm$  SD of 3 determinations, \* $P < 0.05$  vs. the control and # $P < 0.05$  vs. the quercetin-treated cells. (D) Effect of z-VAD-fmk on the quercetin-induced PARP cleavage.

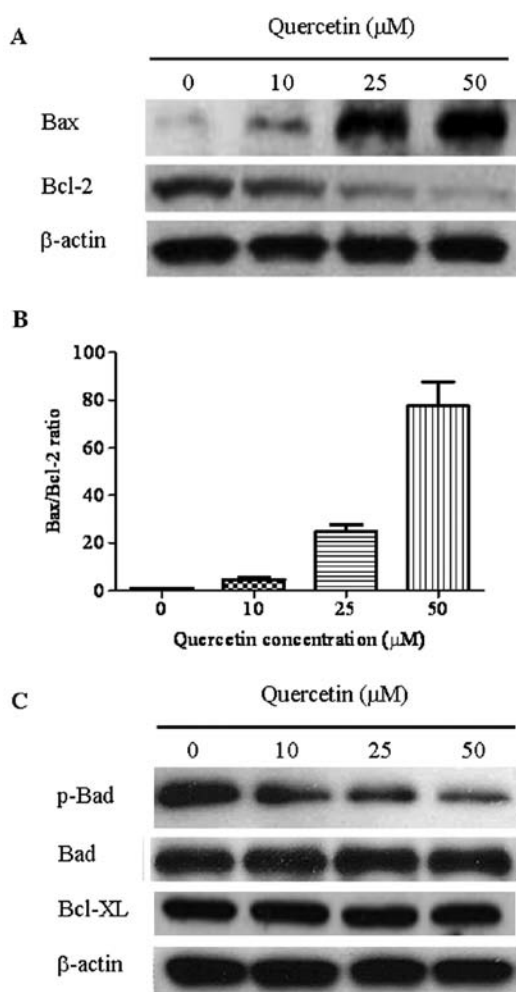


Figure 5. Expression of Bcl-2 family proteins in quercetin-treated U2-OS/MTX300 cells. (A) U2-OS/MTX300 cells were treated with/without quercetin for 48 h. After treatment, the levels of Bcl-2 and Bax were analyzed by Western blot analysis. (B) The ratio of the Bax and Bcl-2 protein expression was shown as mean  $\pm$  SD from 3 independent experiments. \* $P < 0.05$  vs. the control. (C) Western blot analyses for p-Bad, Bad and Bcl-xL after quercetin treatment.

cleave PARP and other critical cellular proteins. In the present study, quercetin treatment activated caspase-3 (Fig. 4A). Meanwhile, the level of cleaved-PARP was also highly increased (Fig. 4B). However, this activation was inhibited by pretreatment with the caspase inhibitor, z-VAD-fmk (Fig. 4C and D). These data indicate that activation of caspase-3 likely played a critical role in quercetin-induced apoptosis in U2-OS/MTX300.

**Effects of quercetin on the Bcl family proteins.** To better understand the underlying molecular forces promoting the quercetin-induced apoptosis in U2-OS/MTX300 cells, protein expression of Bax, Bcl-2, pBad, Bad and Bcl-xL were examined by Western blotting. Following a 48-h treatment with quercetin at doses of 0, 10, 25 or 50 μM, we observed a significant decrease in Bcl-2 accompanied by an inversely-correlated and dose-dependent increase in the protein levels of Bax (Fig. 5A), and an increase in the Bax/Bcl-2 ratio (Fig. 5B). Although there were no observable changes of total Bad and Bcl-xL levels, the down-regulation of p-Bad was detected

(Fig. 5C). These results suggest that quercetin could induce apoptosis in U2-OS/MTX300 cells by affecting the Bcl family proteins.

**Dephosphorylation of Akt was involved in quercetin-induced apoptosis in U2-OS/MTX300 cells.** Bad is a downstream target of Akt and the phosphorylation of Bad is important to the integrity of the mitochondrial membrane system. Therefore, the phosphorylation of Akt was analyzed. As predicted, a dose-dependent dephosphorylation of Akt was observed after quercetin treatment (Fig. 6A). To further confirm the involvement of Akt in the quercetin-induced apoptosis, the U2-OS/MTX300 cells were treated with/without quercetin following the transduction of constitutively active Akt (pLNCX-Myr-Akt) and empty vector (pLNCX). The constitutively active Akt protected against quercetin-induced dephosphorylation of Akt, Bad and PARP cleavage (Fig. 6B). On the other hand, LY294002 (a PI3K inhibitor) used to inhibit the activity of Akt and quercetin were combined together to treat U2-OS/MTX300 cells. We found that LY294002 could significantly enhance the quercetin-induced dephosphorylation of Akt, Bad and the degradation of PARP (Fig. 6C). These results suggested that dephosphorylation of Akt was involved in the quercetin-induced apoptosis.

## Discussion

Despite continuing efforts to improve chemotherapeutic response in osteosarcoma, the development of drug resistance remains a challenge. In fact, only 50-60% of tumors are chemosensitive (3), indicating the dismal outcome that occurs far too often in this disease. One potential strategy to overcome known chemoresistance in osteosarcoma is to seek out alternative anti-cancer agents, particularly those emerging from natural medicine (22).

Quercetin is an all-natural chemical found in many types of plants and food which shows promising anti-cancer activity in several studies, including a phase I clinical trial (23). *In vitro* studies confirm that quercetin at a range of 10-100 μM inhibits cell viability, likely through cell cycle arrest, and induces apoptosis (8-20). While quercetin has been evaluated in several tumors, very little is known about its effects in MTX-resistant osteosarcoma. In this study, we provide evidence that quercetin acts as a potent anti-cancer drug, reduces cellular viability and initiates apoptosis in MTX-resistant osteosarcoma. There was no cross-resistance between MTX and quercetin in U2-OS/MTX300 cells. The 48 h  $IC_{50}$  of quercetin treatment in our model was  $22 \pm 4$  μM and apoptosis was confirmed by Hoechst staining and flow cytometric analysis. These results are consistent with previous studies.

Although the exact mechanism of quercetin-induced apoptosis is still unclear, possible explanations include: down-regulation of the epidermal growth factor receptor (EGFR) family (8,9), promotion of TRAIL-induced apoptosis (10), activation of the AMPK signaling pathway (11), up-regulation of Bax and post-translational regulation of Bcl-2 (14), activation of the intrinsic apoptosis pathway (15,17,20), down-regulation of Mcl-1 and activation of Bax (16), suppression of the PI3K/Akt/IKK $\alpha$ /NF- $\kappa$ B pathway (19) and induction of histone acetylation (24). Given the vast effects of quercetin in various cell

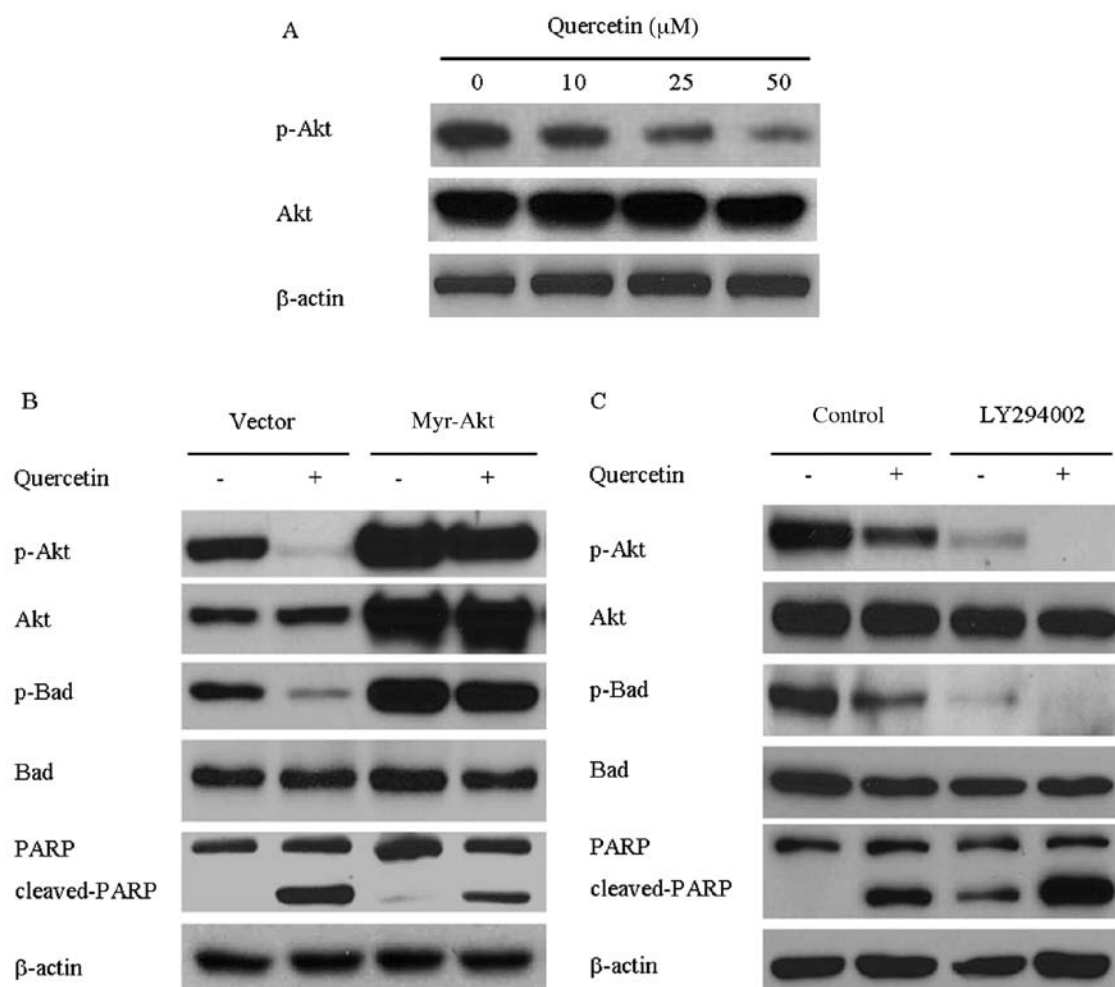


Figure 6. Dephosphorylation of Akt was involved in quercetin-induced apoptosis. (A) U2-OS/MTX300 cells were treated with quercetin for 48 h. After the treatment, cell lysates were extracted, and the levels of p-Akt and Akt were analyzed by Western blot analysis. Quercetin treatment induced obviously dephosphorylation of Akt. (B) Constitutively active Myr-Akt and control vector-transduced U2-OS/MTX300 cells were treated with/without quercetin and the p-Akt, Akt, p-Bad, Bad, PARP and cleaved-PARP were detected by Western blotting. (C) Western blot analyses for p-Akt, Akt, p-Bad, Bad, PARP and cleaved-PARP in U2-OS/MTX300 cells treated with quercetin (25  $\mu$ M) and LY294002 (5  $\mu$ M).

types, its exact molecular mechanism for inducing apoptosis may be cell-type specific.

Mitochondria have been reported to play a critical role in the intrinsic mechanisms of apoptosis (25). The loss of mitochondrial membrane potential facilitates the opening of the outer membrane pore and results in cytochrome c translocation from the mitochondria to cytosol (26). In the present study, progressive deterioration of the mitochondrial membrane potential and subsequent release of cytochrome c into the cytosol was observed after quercetin treatment. In addition, many studies have suggested that caspase-3 can be activated through mitochondria-dependent signaling proteins by the release cytochrome c into the cytoplasm (27). Caspase-3 is classified as an executioner caspase due to its ability to cleave numerous critical cellular proteins including PARP, and generally once activation of caspase-3 has occurred, the cell is committed to death via apoptosis. Our data showed that caspase-3 was highly activated after quercetin treatment. Indeed, pre-incubation with z-VAD-fmk, a caspase inhibitor, effectively decreased the caspase-3 activation. Results of the present study reinforce the idea that quercetin-induced apop-

toxis in U2-OS/MTX300 cells occurs through mitochondria dependent caspase activation.

Previous research has shown that mitochondrial-mediated apoptosis is facilitated by the Bcl-2 homology family proteins (25). This family includes pro-apoptotic proteins, such as Bax and Bad, as well as anti-apoptotic proteins like Bcl-2 and Bcl-xL. The ratio of pro- and anti-apoptotic proteins such as Bax/Bcl-2 is thought to determine, at least in part, the susceptibility of cells to a death signal (27). In the present study, quercetin-induced apoptosis in U2-OS/MTX300 cells was accompanied by down-regulation of Bcl-2 and up-regulation of Bax, resulting in a significant increase of Bax/Bcl-2 ratio. No observable changes in Bad and Bcl-xL were detected, however, significant down-regulation of p-Bad was observed. Previous studies have shown that the apoptotic stimuli can dephosphorylate Bad and release it from the 14-3-3 protein (28,29). Consequently, Bad will compete with Bcl-2/Bcl-xL in binding to Bax (28,29). Therefore, Bax is released and facilitates cytochrome c translocation to trigger the cell death sequence (30). Taken together, our results suggest that the quercetin-induced apoptosis in U2-OS/MTX300 is regulated

by a balance between the pro- and anti-apoptotic Bcl-2 family proteins within the cells.

To further understand the mechanism of quercetin-induced apoptosis in U2-OS/MTX300 cells, the role of Akt, a central regulatory protein of cell survival, in this process was investigated. Many experiments have shown that functional Akt could phosphorylate Bad at Ser136 to promote the stabilization of the mitochondrial membrane system and cell survival (30). In our study, Akt was obviously dephosphorylated after quercetin treatment. Insertion of constitutively active Akt could attenuate the dephosphorylation of Akt, Bad and the degradation of PARP induced by quercetin. Moreover, combination of quercetin and LY294002 remarkably enhanced the quercetin-induced dephosphorylation of Akt, Bad and the cleavage of PARP. These data strongly suggest that quercetin-induced apoptosis was closely associated with dephosphorylation of Akt.

In conclusion, the present study provides evidence that quercetin is a potent anti-cancer agent in MTX-resistant osteosarcoma cells U2-OS/MTX300. Quercetin-induced apoptosis in U2-OS/MTX300 occurred through the mitochondrial-dependent pathway and dephosphorylation of Akt. These data indicate that quercetin may have merit as a potential chemotherapeutic agent for MTX-resistant osteosarcoma.

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