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Fisetin, a dietary flavonoid induces apoptosis via modulating the MAPK and PI3K/Akt signalling pathways in human osteosarcoma (U-2 OS) cells

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Introduction

Osteosarcoma, most frequent of the bone tumors, occurs predominantly in adolescents and children. It is characterized by high recurrence and metastasis of bone and soft tissue (Kager et al., 2003; Kim et al., 2004). Treatment includes multimodal approaches including surgery, radiotherapy and chemotherapy (Guijarro et al., 2014; Luetke et al., 2014). However, drug resistance and severe side effects associated with conventional therapeutics (Chou and Gorlick, 2006) have urged for novel and more effective therapeutics.

Studies have reported dysregulation of several signalling pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Zhang et al., 2015). PI3K/Akt pathway is negatively regulated by phosphatase and tension homolog (PTEN) can activate downstream targets such as mammalian target of rapamycin (mTOR) (Zhang et al., 2015). As a vital pathway in various physiological and pathological processes it is well established in human cancers (Yuan and Cantley, 2008; Porta et al., 2014).

Extracellular signal regulated kinase (ERK1/2), c-Jun N-terminal (JNK) and p38 MAPK, main members of the mitogen-activated protein kinases (MAPKs) family regulate several of cellular responses (Genestra, 2007; Wagner and Nebreda, 2009). MAPK cascades exert crucial roles in drug-induced apoptosis in osteosarcoma (Chen et al., 2009; Noh et al., 2011). Evidences suggest that cancer cells are under increased oxidative stress (Pelican et al., 2004). Further excessive reactive oxygen species (ROS) affect MAPK signals (Shen and Liu, 2006; Avissiti et al., 2014). Accordingly, targeted inhibition of the MAPK and PI3/Akt pathways may be effective in cancer treatment.

Studies have demonstrated anti-cancer effects of various phytochemicals (Lin et al., 2011; Li et al., 2014,
Materials and Methods

Cell lines

The human osteosarcoma cell line U-2 OS (HTB-96TM, ATCC) was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 μg/mL), 2 mM glutamine and penicillin (100 U/mL) at 37°C in a humidified atmosphere with 5% CO₂.

Reagents and chemicals: Fisetin, glutamine, RPMI1640 medium, fetal calf serum (FCS), penicillin, streptomycin, PBS and 0.25% trypsin were purchased from Sigma Aldrich (St.Louis, MO, USA). Antibodies against ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, phospho-JNK, c-Jun, phospho-c-Jun, Akt, phospho-Akt, GSK3β (glycogensynthase kinase 3β), phospho-GSK3β), NF-κB, IκB, caspase-3, caspase-8, caspase-9 (Cell Signaling Technology, Beverly, MA), Bcl-xl, Bcl-2, Bad, Bax, mTORC1, cyclinD1 and PTEN (Santa Cruz, Biotechnology, Inc. Santa Cruz, CA, USA) were used in the study. Chemicals and reagents used in the study were obtained from Sigma Aldrich (St.Louis, MO, USA) unless otherwise specified.

Cell viability assay

The anti-proliferative effect of fisetin on osteosarcoma cells was determined using MTS kit (Promega, Madison, USA). Briefly, cells at a density of 3-7 × 10³ were seeded per well in 96-well plates. After 12 hours of incubation at 37°C, the cells were treated with various concentrations of fisetin (20-100 μM) for 12, 24 or 48 hours. The cells were then incubated with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and phenazine methosulfate (PMS) for 2-4 hours at 37°C as per manufacturer’s instructions. The color developed was read at 490 nm using a MR7000 microplate reader (Dynatech, USA).

Analysis of apoptosis by annexin V assay

Following incubation with various concentrations of fisetin (20-100 μM) for 24 and 48 hours, cells were trypsinized and collected for detection of apoptosis using annexin V-FITC (Fluorescein isothiocyanate) apoptosis detection kit (Santa Cruz Biotechnology, USA). Briefly, 1 x 10⁶ cells treated with fisetin were subjected to annexin V staining. The cells were washed in PBS and resuspended in binding buffer containing (100 μL) FITC-conjugated anti-annexin V antibody and analyzed for fluorescence using a flow cytometer (FACS Calibur, BD Biosciences).

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was measured with JC-1 fluorescent probe (Cayman Chemical). In brief, 2 x 10⁵ cells exposed to fisetin (20 -100 μM) for 24 hours were incubated with JC-1 for 20 min at 37°C. The stained cells were washed twice with PBS and analyzed by a flow cytometer. Mitochondrial depolarization was indicated by a decrease in the red/green fluorescence intensity ratio.

Measurement of ROS

Generation of intracellular ROS was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Beyotime). Briefly, 2 x 10⁵ cells were plated in each well of six-well plates. Following incubation with various concentrations of fisetin (20-100 μM) for 12 hours and 24 hours, cells were incubated with DCFH-DA (10 μM) for 30 min at 37°C. The level of ROS was determined by fluorescence microscopy and flow cytometer (FACS Calibur, BD Biosciences).

Western blotting

The U-2 OS were seeded at a density of 5 x 10⁶ cells in 60-mm dishes and exposed to fisetin (20 -100 μM) for 24 hours. The cells were collected by centrifugation and the pellets were lysed in RIPA lysis buffer containing protease inhibitor cocktail for 30 min on ice. To determine the cytoplasmic IκB, cytoplasmic extracts were prepared (Lee et al., 2006). To analyze NF-κB (p65), nuclear extract was prepared using a previously described method (Lee et al., 2006). Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). Proteins were then separated by SDS-PAGE, electro-transferred to nitrocellulose membranes, blotted with respective antibodies (Cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, ERK1/2, p-ERK1/2, p38, p-p38, JNK, p-JNK, c-Jun, p-c-Jun, Akt, phospho-Akt, GSK3β, phospho-GSK3β, NF-κB (p65), IκBα, Bcl-xl, Bcl-2, Bad, Bax, mTORC1, cyclinD1, PTEN and β-actin). The immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare).

Statistical analysis

The data are presented as means ± SD obtained from three or six individual experiments. The values were analysed by one-way ANOVA (analysis of variance).
All statistical analyses were performed using the SPSS software (version 17.0, SPSS, USA).

Results

Fisetin induces apoptosis of osteosarcoma cells

U-2 OS osteosarcoma cancer cells exposed to fisetin showed considerable susceptibility to different concentrations. The cell viability gradually decreased in a concentration and time-dependent manner. Treatment with fisetin at 80 and 100 µM presented more pronounced decreases in cell viability than lower doses (Figure 1).

To further assess apoptosis induced by fisetin, annexin-V/PI staining followed by FACS analysis was performed. Annexin V/PI staining was used to analyze the apoptosis of U-2 OS cells exposed to different concentrations of fisetin. The results showed that fisetin induced apoptosis in a dose-dependent manner (Figure 2).

Figure 1: Effect of fisetin on the cell viability of U-2 OS cells

Values are represented as mean ± SD; n=6; *represents p<0.05 compared with control as determined by one way-ANOVA

Figure 2: Influence of fisetin on apoptotic cell counts

Fisetin induced apoptosis of the U-2 OS cells in a dose-dependent manner as determined by Annexin V staining. Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one way-ANOVA
performed. Fisetin caused significant (p<0.05) increases in apoptotic cell counts (Figure 2). Fisetin at 100 µM exhibited more profound effects than the lower concentrations. Moreover, the time of exposure to fisetin also influenced the effects. Incubation with fisetin for 48 hours brought about more apoptosis than 24 or 12 hours of exposure. Significant (p<0.05) difference were observed between the apoptotic cell counts following 24 and 48 hours of fisetin treatment.

Effects of fisetin on apoptotic protein expression

Caspases-3,-9 and -8 were down-regulated in U2-OS cells, suggesting suppression of apoptosis in cancer cells. However, fisetin effectively caused significant (p<0.05) up-regulation in the expression of cleaved caspase -3,-8 and -9, thus promoting the apoptotic cascades (Figure 3). Further, fisetin at 100 µM dose was more effective in enhancing the expression of caspases than 20-60 µM. Significant (p<0.05) increase in the levels of Bcl-2 and Bcl-xL expressions in the U-2 OS cells was observed, nevertheless following fisetin exposure caused significant down-regulation in the expression. In addition, the level of pro-apoptotic proteins such as Bax and Bad that mainly modulate apoptosis, were enhanced observably in the U2-OS cells exposed to fisetin. Up-regulation of Bax and Bad correlated with the down-regulation of inhibitors of apoptosis- Bcl-2 and Bcl-xL.

Decrease in mitochondrial transmembrane potential indicates early apoptosis. Fisetin exposure resulted in a noticeable decline of mitochondrial transmembrane potential (ΔΨm) in a dose-dependent manner (Figure 4). Bcl-2 and Bcl-xL proteins modulate apoptosis at the mitochondrial outer membrane and control the initiation of mitochondrial outer membrane permeabilization (Anilkumar and Prehn, 2014). In our study, Bcl-

Figure 3: Influence of fisetin on the expression of apoptosis pathway proteins

(A) Fisetin markedly up-regulated the pro-apoptotic proteins and reduced the expressions of Bcl-2 and Bcl-xL. (B) Relative expression of the proteins. Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one way-ANOVA
Figure 4: Effect of fisetin on the mitochondrial membrane potential of U-2 OS cells
Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one way-ANOVA

Figure 5: Fisetin regulates the expression of PI3K/Akt pathway proteins
(A) Fisetin effectively modulates the expressions of the PI3K/Akt signalling proteins. Fisetin dose-dependently inhibits the pathway. (B) Relative expressions of the PI3K/Akt pathway proteins. Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one-way ANOVA
xL and Bcl-2 were down-regulated in a concentration-dependent manner after fisetin treatment. This could have caused alteration in the mitochondrial membrane potential and thus promoting apoptosis.

**Effect of fisetin on PI3/Akt pathway proteins**

Activation of the PI3K/Akt pathway plays a critically oncogenic role in the initiation and progression of OS (Zhang et al., 2015). Effect fisetin over the expressions of PI3/Akt pathway proteins in U2-OS cells were assessed. Akt is a critical signalling junction downstream of the PI3K pathway which is activated through phosphorylation. p-Akt levels are raised in OS cells, whereas PTEN, an important regulator of the pathway was found to be down-regulated in U2-OS cells not exposed to fisetin (Figure 5). However, fisetin at various concentrations (20-100 µM) caused a significant (p<0.05) decrease in the level of p-Akt and mTORC1 (an important effector protein of Akt), while up-regulated PTEN. Level of phosphorylated glycogen synthase kinase β (GSK3β), (a serine/threonine kinase) and cyclin D1 were potentially decreased by fisetin which is in line with raised non-phosphorylated levels of GSK3β. Another important target activated by Akt is nuclear factor-xB (NF-xB), the levels of which are raised in OS cells. Down-regualtion of NF-xB along with significant up-regulations in IxB upon fisetin treatment correlates with the down-regulation of p-Akt levels. These observations suggest the effective blocking of the PI3K/Akt pathway, an important target in cancer therapy.

**Influence of fisetin on generation of ROS**

It is well documented that excessive generation of ROS could interfere with DNA, lipids and proteins and cause cellular damage (Simon et al., 2000; Chen et al., 2007). U-2 OS cells presented raised levels of ROS. Fisetin exposure at various concentrations resulted in a noticeable increase in ROS levels in a dose-dependent way (Figure 6). This increase though observable, was not significant.

**Fisetin modulates the ERK/JNK/p38MAPK signalling cascade**

As MAPKs are important regulators of stress responses including the induction of apoptosis (Johnson and Lapadat, 2002) we assessed the influence of fisetin over JNK, ERK1/2 and p38 expression level. Significant up-regulation in the phosphorylated levels of JNK was observed (Figure 7). Incubation of OS cells in the presence of fisetin (20-100 µM) considerably (p<0.05) down-regulated the activation and decreased the levels of p- ERK1/2 and ERK1/2 as well. However, higher expression of phosphorylated p38 was observed. Further, fisetin brought multi-fold raise in the levels of p-c-Jun. While 20-60 µM of fisetin was effective in decreasing the expression of the phosphorylated forms of ERK1/2, 100 µM fisetin presented additional down-regulation.

**Discussion**

Most cancer therapeutic regimens including chemotherapy, inhibit tumors by activating apoptosis (Zhang et al., 2013). In our study, dietary flavonoid, fisetin at concentrations 20-100 µM induced a decrease in the viability of U-2 OS cells. Further, increase in apoptotic cell counts were observed following fisetin exposure as determined by annexin-V/PI staining.

Mitochondria play a crucial role in apoptosis (vanLoo et al., 2002). Disruption of mitochondrial integrity has been established as one of the earliest intracellular events that occur following initiation of apoptosis (Qi et al., 2010). Decreases in the mitochondrial transmembrane potential (Δψm) is associated with mitochondrial dysfunction, that activates the efflux of cytochrome C to

![Figure 6. Influence of fisetin on intracellular ROS generation in U-2 OS cancer cells](image-url)
the cytosol and initiates the caspase cascade leading to apoptosis (Han et al., 2006; Kroemer et al., 2007). Therefore, the observed decrease of Δψm following treatment with fisetin, suggests mitochondrial dysfunction and disruption in mitochondrial integrity that could have possibility be responsible for the raised apoptotic cell counts.

To assess the activation of caspase cascade, expression levels of caspases-9, -8 and -3 were determined. Activation of caspase-9 and -8 suggests the involvement of both intrinsic and extrinsic pathways of apoptosis that sequentially lead to the activation of caspase-3 (Hartojo et al., 2010). Fisetin was observed to cause dose-dependent multifold activation of caspase-3, -8 and -9. These findings indicate that fisetin induces apoptosis in human osteosarcoma cells by activating caspase cascades.

Bcl-2 family proteins are involved in the regulation of apoptosis and mitochondrial membrane potential (Hunter et al., 2007; Heath-Engel et al., 2008; Gyrd-Hansen and Meier, 2010; Huttemann et al., 2011). The pro-apoptotic Bcl-2 family proteins such as Bax and Bak form pores in the outer mitochondrial membrane and stimulate apoptosis, while the anti-apoptotic proteins including Bcl-2 and Bcl-xL inhibit pore formation (Gross et al., 1999). It has been demonstrated that Bcl-xL...
has been highly expressed in some hematopoietic and solid tumors (White et al., 2005). Down-regulation of Bcl-xL and Bcl-2 observed in fisetin exposure could have contributed to disruption of the membrane integrity leading to intrinsic apoptosis (Gottlieb et al., 2000). Down-regulation of Bcl-xL was accompanied with a marked up-regulation in the expression of the pro-apoptotic proteins Bax and Bad, suggesting that fisetin modulates the expressions of not only caspases but also the apoptotic proteins to trigger apoptosis in the osteosarcoma cells.

MAPK signalling cascades are important regulators of stress responses, including the induction of apoptosis (Johnson and Lapadat, 2002; Park, 2011; You and Park, 2011). Previous studies have suggested that JNK, p38, and ERK1/2 pathways have critical roles in the induction of apoptosis (Tournier et al., 2000; Kim and Chung, 2008). Activation of ERK has been shown to promote proliferation and survival of most of the cell types (Lewis et al., 1998; Wang et al., 1998) and as well regulate cell apoptosis (Ishikawa and Kitamura, 1999). Nevertheless, the MAP kinases- JNK and p38 are often activated by oxidative stress and xenobiotics, and have been reported to subsequently induce apoptosis (Lewis et al., 1998; Obata et al., 2000). Studies suggest that p38 and/or JNK directly activate the caspase cascade, and also cause the activation of the apoptotic transcription factor c-Jun (Davis, 2000). In our study, fisetin brought about marked down-regulation of the phosphorylated forms of ERK1/2, whereas up-regulated the phosphorylated levels of JNK, and p-p38 in a dose-dependent manner. This observed activation of JNK could have caused up-regulation of p-c-Jun in the U-2 OS cells.

Excessive generation of ROS could interfere with cellular signalling pathways (Simon et al., 2000; Trachootham et al., 2009) and is one of the contributing factors in the malignant transformation of normal cells via inducing oxidative DNA damage (Klaugui et al., 2010; Lee et al., 2012). However, induction of ROS plays a significant role in the chemotherapeutic activity of several anticancer drugs and anticancer compounds (Fruehauf and Meyskens, 2007; Trachootham et al., 2009). Drug-induced ROS mediates the activation of MAP kinases, disrupts the mitochondrial membrane potential and subsequently activates apoptotic caspases in cancer cells (Zhang et al., 2000; Raza et al., 2011). JNK could be activated by stimuli as cytokines, ROS, pathogens, toxins, drugs, and metabolic changes (Seki et al., 2012). Thus the observed raised levels of ROS could have also contributed to the activation of JNK leading to apoptosis. Fisetin thus could have promoted the generation of ROS and caused the activation of JNK via ROS-induction or could have acted directly.

PI3K/Akt pathway is another major signalling cascade that is deregulated in many cancers (Porta et al., 2014). Dysregulation of this pathway plays a vital role in multiple pathological processes of OS including cell cycle progression, tumorigenesis, invasion, angiogenesis, metastasis, apoptosis and chemoresistance (Zhang et al., 2015). Activation of PI3K leads to the activation of major effector Akt through phosphorylation. Activated Akt translocates to the cytoplasm and nucleus and further cause phosphorylation of many downstream effector proteins that regulate various cellular functions such as mTOR and GSK3β, which further leads to cell cycle progression. Additionally, Akt increases the activity of inhibitor of κB (IκB) kinase (IKK), that leads to phosphorylation and thus degradation of IκB causing the subsequent release of NF-κB, a central signalling factor that has been reported to be involved in tumorigenesis of various cancers (Ahmad et al., 2013).

Fisetin at various concentrations caused down-regulation of p-Akt in a dose-dependent way. This decrease in the activation of p-Akt could have contributed the reduced expression levels of p-GSK3β that further inhibited cyclin D1 expression. Moreover, fisetin effectively inhibited NF-κB and as well modulated the levels of IκB and mTORC1. PTEN levels were enhanced significantly by fisetin. PTEN is a main negative regulator of the PI3K/Akt pathway, and loss of PTEN activity has been frequently observed in OS (Nielsen-Preiss et al., 2003). Thus, PTEN activators may be an alternative approach for suppression of the pathway in OS. Fisetin induced PTEN levels contribute to the negative regulation of the PI3K/Akt pathway thus reducing cell cycle progression and promoting apoptosis.

Conclusion
Fisetin at 20-100 µM concentration potentially induced apoptosis of the U-2 OS cells by up-regulating the apoptotic proteins and also modulating the expression of MAPK and PI3K/Akt signalling cascades proteins.

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Conflict of Interest
Authors declare no conflict of interest

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