

ORIGINAL ARTICLE

Anticancer activity of Fisetin against the human osteosarcoma cell lines involves G2/M cell cycle arrest, mitochondrial apoptosis and inhibition of cell migration and invasion

Chunyang Xing¹, Yuzhu Zhang¹, Rong Su², Ronghuan Wu¹

¹Department of Orthopaedics, the First Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang, China, 310003; ²Key Laboratory of Combined Multi-organ Transplantation, the First Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang, China, 310003.

Summary

Purpose: Osteosarcoma is rare but fatal type of human malignancy. The high metastasis rate, late diagnosis, emergence of drug resistance against drugs such as doxorubicin, and the lack of therapeutic targets obstructs the treatment of osteosarcoma. The present investigation explores the anticancer properties of Fisetin against human osteosarcoma cells.

Methods: The cell viability was determined by WST-1 assay. DAPI and Annexin V/propidium iodide (PI) assays were used for detection of apoptosis. Flow cytometry was used for the determination of osteosarcoma MG-63 cell distribution. Wound healing and transwell assays were used for cell migration and invasion. Western blotting was used for protein expression analysis.

Results: The results showed that Fisetin inhibits the growth of the MG-63 cells in a dose-dependent manner. Fisetin showed an IC₅₀ of 18 μ M against the MG-63 cells. The growth

inhibitory effects of Fisetin were mainly due to induction of apoptosis which was accompanied by enhancement of the caspase-3 and Bax and depletion of Bcl-2 expression. Fisetin treatment increased reactive oxygen species (ROS) from 100 in untreated to 220% at 36 μ M and decreased mitochondrial membrane potential (MMP) levels from 100 in untreated to 21% at 36 μ M. Fisetin also induced G2/M cell cycle arrest of the MG-63 cells and suppressed the expression of cyclin-B1. The wound healing and the transwell assay showed that Fisetin suppressed the migration and invasion of the MG-63 cells.

Conclusions: Taken together, Fisetin may find use as lead molecule in the osteosarcoma therapeutic development programmes.

Key words: osteosarcoma, apoptosis, cell cycle arrest, invasion, fisetin

Introduction

Among the plant secondary metabolites, flavonoids are the most prevalent diverse and ubiquitously distributed group [1]. More than 5000 flavonoids have been reported from plants and the number is still growing. These metabolites are abundantly found in foods and are thus considered safe for human consumption. Because of their stability, they are not lost during cooking [3]. Pharmacologically, flavonoids are very important and have

been reported to exhibit a wide diversity of bioactivities [4]. The anticancer properties of flavonoids are very well reported in the literature [5]. Fisetin is an important flavonoid and has been extracted from different plant species. It has been shown to exhibit antioxidant, anti-inflammatory and anticancer properties [6]. Nonetheless, the anticancer properties of Fisetin against human osteosarcoma cells have not been reported till today. This study

Corresponding author: Ronghuan Wu, MD. Department of Orthopaedics, the first affiliated Hospital, Zhejiang University, 79 Qingchun Rd, Hangzhou, Zhejiang, China, 310003.
Tel and Fax: +86 0571 87236114, Email: wuronghuan@zju.edu.cn
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for the first time investigates the anticancer properties of Fisetin against human osteosarcoma cells. Osteosarcomas are generally primary malignant tumors of bone that are characterised by osteoid or immature bone development by the malignant cells [7]. Osteosarcoma is considered as one of the rare cancers accounting for around 1% of all the cancers detected in United States. Among all age groups, osteosarcomas are more prevalent in children and adolescents and in United States, around 55% of all the osteosarcomas occur in children and adolescents [8]. Osteosarcomas are considered fatal types of malignancies owing to their frequent ability to develop metastasis. It has been reported that around 80% of osteosarcomas give rise to metastasis and hence there is urgent need for early detection, identification of efficient therapeutic targets and effective chemotherapy for the treatment of this disease [9]. Herein, we report that Fisetin inhibits the growth of human osteosarcoma cells via multiple mechanisms which include induction of apoptosis, loss of mitochondrial potential and triggering cell cycle arrest. Taken together, Fisetin can potentially be utilised as a lead molecule for the development of osteosarcoma therapy

Methods

Cell culture conditions

The osteosarcoma MG-63 cell line and the normal human bone cell line hFOB1.19 were obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Massachusetts, USA), 100 µg/ml streptomycin and 100 U/ml penicillin G (Himedia, Pennsylvania, USA) in an incubator at 37°C with 5% CO₂.

WST-1 assay

The proliferation rate of MG-63 cells was monitored by WST-1 assay. Briefly, MG-63 cells were cultured in 96-well plates at the density of 2×10^5 cells/well and incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for another 4 h. The absorbance was then taken at 450 nm using a Victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals.

DAPI and Annexin V/PI staining

The MG-63 cells were cultured for 24 h at 37°C and then fixed with ethanol (70%) for 20 min. The cells were then subjected to PBS washing and subsequently stained with DAPI. Finally, the cells were examined under microscope to detect the induction of apoptosis. The MG-63 cells were transfected with appropriate constructs and then incubated for 48 h at 37°C. The cells were then dissociated with trypsin and then PBS-washed. The cells

were then resuspended in 1X binding buffer which was followed by the addition of 5 µL of annexin V-FITC and propidium iodide (PI). The cell culture was then placed in the dark for 15 min. The apoptosis percentage was then evaluated by a flow cytometer.

Cell cycle analysis

The Fisetin-treated MG-63 cells were harvested and washed twice with PBS. Cells were then fixed with 70% ethanol for ~1 h at -20°C and then washed again with PBS. Cells were resuspended in a solution of PI (50 µl/ml) and RNase1 (250 µg/ml) (Invitrogen Life Technologies, Massachusetts, USA). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting using 10,000 cells/group with a flow cytometer.

Estimation of ROS and MMP

MG-63 cells were seeded at a density of 2×10^5 cells/well in 6-well plates and incubated for 24 h. Cells were then treated with 0, 9, 18 and 36 µM Fisetin for 24 h at 37°C in 5% CO₂ and were washed twice with PBS and resuspended in 500 µl dihydrofluorescein diacetate (10 µM) (Sigma-Aldrich USA) for ROS estimation and DiOC6 (1 µmol/l) at 37°C in the dark for 35 min to measure the MMP.

Cell migration and invasion assay

Transwell chambers with Matrigel were employed to monitor the MG-63 cell invasion. In brief, the cells were cultured for 24 h and then harvested and suspended

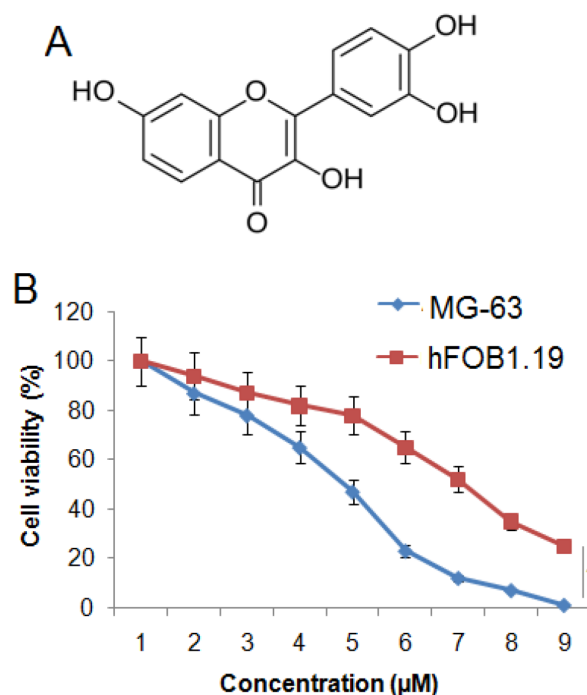


Figure 1. A: Chemical structure of Fisetin. **B:** WST-1 assay showing the effect of Fisetin on the viability of osteosarcoma MG-63 and normal hFOB1.9 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

in fresh Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Massachusetts, USA), while 200 μ L of the cell suspension containing approximately 5×10^4 cells was placed onto the upper compartment and fresh 500 mL medium was placed in the lower compartment. After 24 h the cells present at the upper compartment were removed by swabbing, while cells that invaded to the lower surface were fixed and then subsequently stained with 0.05% crystal violet. Finally, 10 random fields were selected to determine the invasion under light microscope. The cell migration was determined by a similar procedure except that Matrigel was not used.

Western blotting

The osteosarcoma cells were lysed and the protein concentration in each sample was measured by Bradford assay. Equal concentrations of the proteins from each sample were loaded on 10% SDS polyacrylamide gels which was followed by shifting to polyvinylidene fluoride membranes. Blocking of the membrane was then performed by fat-free milk (5%). This was followed by incubation with a primary antibody for 24 h at 4°C. Subsequently, a secondary antibody was added at 25°C for about 2 h. The bands of interest were finally observed by chemiluminescence.

Statistics

Statistical analyses were performed using a one way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test using SPSS software package v9.05 (SPSS, Inc., Chicago, IL, USA). Data are presented as mean \pm standard deviation, and $p < 0.05$ was considered to indicate a statistically significant difference.

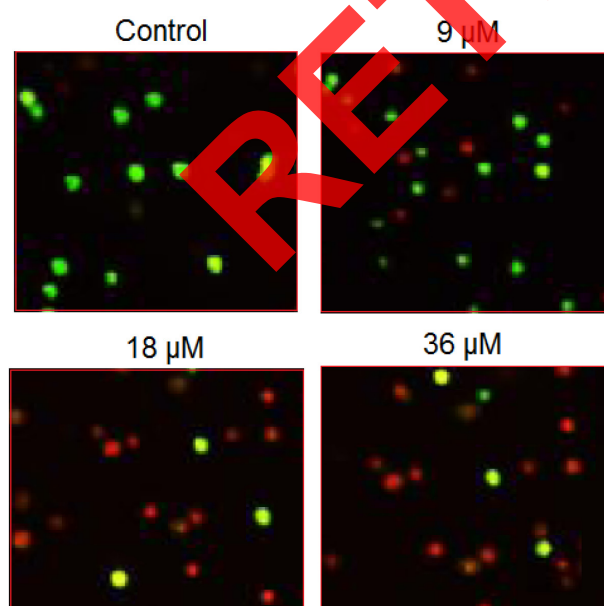


Figure 2. AO/EB staining showing the induction of apoptosis in the Fisetin-treated MG-63 cells. Green colour indicates normal cells, Orange color indicates early apoptotic and red color cells indicate late apoptotic cells. The experiments were performed in triplicate.

Results

Growth inhibitory properties of Fisetin

The WST-1 assay was used to investigate the growth inhibitory properties of Fisetin (Figure 1A) on the human MG-63 osteosarcoma cells and normal hFOB1.19 cells. The results showed that Fisetin inhibits the growth of the MG-63 cells in a dose-dependent manner. Fisetin showed an IC_{50} of 18 μ M against the MG-63 cells (Figure 1B). The growth inhibitory properties of Fisetin were also assessed on the normal hFOB1.19 bone cells and it was revealed that Fisetin exhibits comparatively lower growth inhibitory effects on the normal cells as evidenced from the IC_{50} of $> 80 \mu$ M.

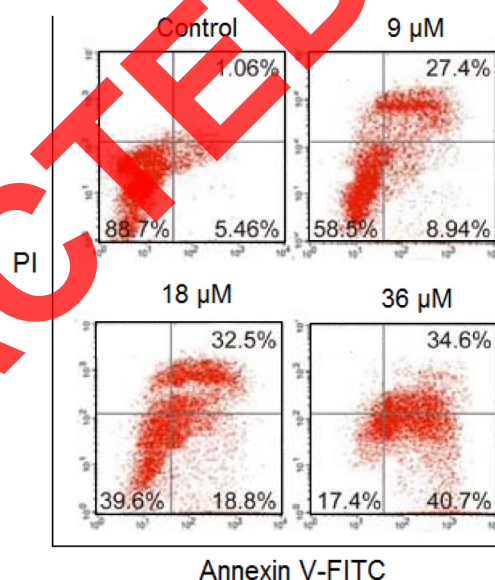


Figure 3. Annexin V/PI staining showing the percentage of apoptosis in MG-63 cells at indicated concentrations of Fisetin. The Figure depicts that the percentage of the apoptotic cells increases with increase in the concentration of Fisetin. The experiments were performed in triplicate.

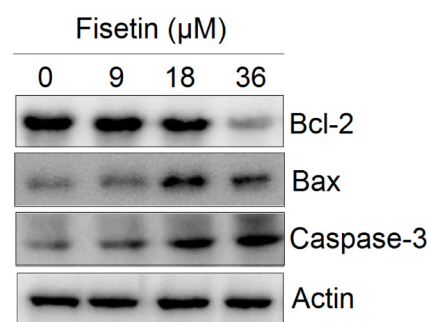


Figure 4. Western blot analysis showing the effect of Fisetin at indicated concentrations on the expression of Bcl-2, Bax and Caspase-3. The Figure depicts that the expression of the Bax and Caspase-3 increases and Bcl-2 decreases with increase concentration of Fisetin. The experiments were performed in triplicate.

Apoptosis-inducing properties of Fisetin

The acridine orange (AO)/ethidium bromide (EB) assay indicated nuclear fragmentation induced by Fisetin in the MG-63 cells, suggestive of apoptosis (Figure 2). The annexin V/PI assay showed apoptosis to be 5.46, 8.94, 18.8 and 40.7% at 0, 9, 18 and 36 μM concentrations of Fisetin, indicative of the dose-dependent apoptosis inducing properties of Fisetin (Figure 3). Western blotting showed en-

hancement of the caspase-3 and Bax and depletion of Bcl-2 expression in MG-63 cells upon Fisetin treatment (Figure 4). The ROS levels were also increased upon Fisetin treatment from 100 to 220% in MG-63 cells, while the MMP levels showed a constant decrease from 100 to 21% (Figure 5A and Figure 5B).

G2/M cell cycle arrest induced by apoptosis

The treatment of MG-63 cells also caused a remarkable increase in the G2/M phase cells, suggestive of the G2/M phase arrest of the MG-63 cells (Figure 6A). Western blot analysis showed that Fisetin caused a considerable depletion in the expression of cyclin B1 in MG-63 cells (Figure 6B).

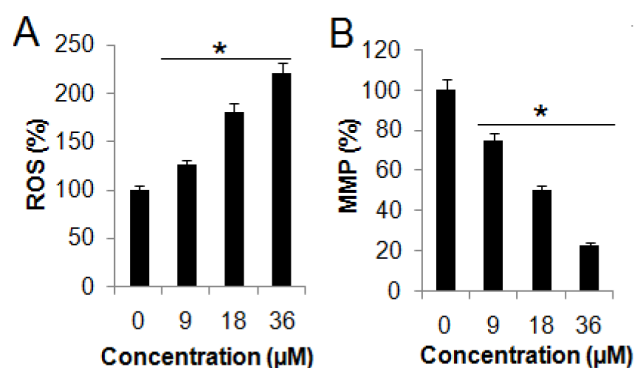


Figure 5. Effect of Fisetin on A: ROS and B: MMP levels of the MG-63 cells at indicated concentrations. The Figure depicts that ROS levels increase and MMP levels decrease with increase in the concentration of Fisetin. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

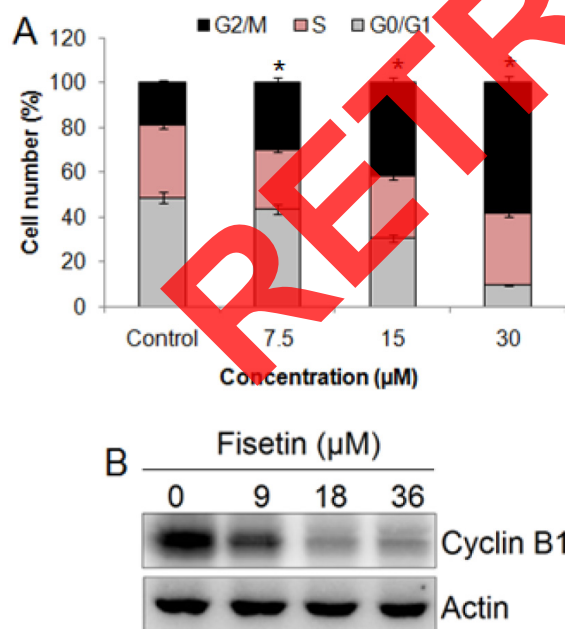


Figure 6. A: Distribution of MG-63 cells in different cell cycle phases at indicated concentrations of Fisetin. The Figure depicts that Fisetin induces G2/M cell cycle arrest of the MG-63 cells. **B:** Western blotting showing the effect of Fisetin on the expression of cyclin B1. The Figure depicts that the expression of cyclin B1 decreases dose dependently. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

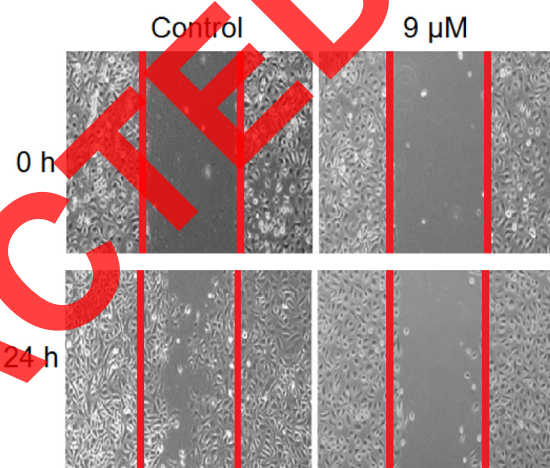


Figure 7. Wound healing assay showing Fisetin inhibits the migration of the MG-63 cells at IC_{50} . The experiments were performed in triplicate.

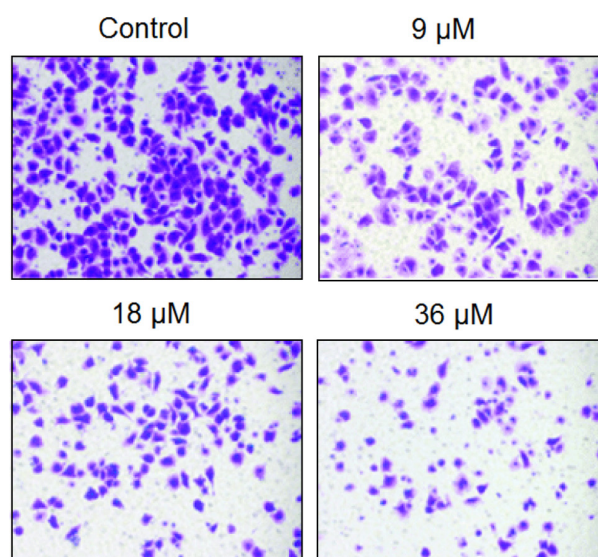


Figure 8. Transwell assay showing the effect of Fisetin on the invasion of the MG-63 cells. The Figure shows that Fisetin suppresses the migration of the cancer cells dose-dependently. The experiments were performed in triplicate.

Inhibition of cell migration and invasion by Fisetin

The wound healing assay showed a remarkable decrease in the migration of the MG-63 osteosarcoma cells upon their treatment with Fisetin at IC₅₀ (Figure 7). The transwell invasion assay also showed a dose-dependent decrease of the MG-63 cells upon Fisetin treatment (Figure 8).

Discussion

Osteosarcoma is one of the rare but fatal diseases, common in children and adolescents [10]. The high metastasis rate, late diagnosis, emergence of drug resistance against the drugs such as doxorubicin, and the lack of therapeutic targets obstructs the treatment of osteosarcoma [11]. Herein, we showed that Fisetin inhibits the growth of the MG-63 osteosarcoma cells and at the same time shows comparatively lower growth inhibitory against the normal hFOB1.19 cells. These observations point towards the selective growth inhibitory properties of Fisetin against the cancer cells. Growth inhibitory effects of Fisetin have also been reported against breast cancer cells [12]. Moreover, previous studies have also shown flavonoids to be safer for human consumption [13]. The apoptosis assays showed that Fisetin inhibits the growth of the MG-63 cells via induction of apoptosis. The apoptosis initiation in the MG-63 cells was also accompanied with enhancement of the caspase-3 and Bax and depletion of Bcl-2. Previous studies also support the present findings as Fisetin has been reported to induce apoptosis in human hepatic and breast cancer cells [12,14]. Also, previous studies have shown that induction of apoptosis may also be associated with significant increase in the ROS production and depletion of the MMP levels [15]. Herein, we also report that Fisetin increased ROS production and decreased the MMP in the MG-63 cells. A study previously carried out showed that

this molecule induces cell cycle arrest in colon cancer cells [16]. Li et al reported Fisetin induced cell cycle arrest in bladder cancer cells [17]. Pal et al reported G2/M cell cycle arrest induced by Fisetin in human epidermoid carcinoma cells [18]. In yet another study Fisetin has been shown to induce cell cycle arrest in acute promyelocytic leukemia cells [19]. In the current study we also found that Fisetin induced G2/M arrest of the osteosarcoma MG-63 cells which was also accompanied by depletion of the cyclin B1 expression. The effects of Fisetin were also monitored on the migration and invasion of the MG-63 cells and it was revealed that this molecule suppressed both the migration and invasion of the human osteosarcoma MG-63 cells. A previous study showed that Fisetin suppresses the migration and invasion of lung cancer cells [20]. In another study, Chou et al reported inhibition of the migration and invasion of the cervical cancer cells by Fisetin [21].

Conclusion

From the findings of the present study, it is concluded that Fisetin exhibits remarkable and selective growth inhibitory effects on the human osteosarcoma cells via induction of apoptosis and cell cycle arrest. Taken together, Fisetin exhibits the potential to be utilised as a lead molecule for further progression of osteosarcoma therapy.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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