

Fisetin inhibits the proliferation of gastric cancer cells and induces apoptosis through suppression of ERK 1/2 activation

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Abstract. The present study aimed to investigate the effect of fisetin on proliferation and apoptosis of gastric cancer cells, as well as the underlying mechanism. Proliferation in SGC7901 cancer and GES-1 normal cells was analyzed using a CCK-8 assay. Apoptosis was analyzed using an Annexin V/Propidium Iodide apoptosis kit and phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 was analyzed by western blot assay. Treatment of SGC7901 cells with various concentrations (1, 5, 10, 15 and 20 μ M) of fisetin for 48 h resulted in a concentration dependent reduction in proliferation. Flow cytometry revealed a marked increase in apoptosis from 5 μ M concentration of fisetin after 48 h. The percentage of apoptotic cells increased to 87% following treatment with 15 μ M fisetin for 48 h, compared with 2% in control. Treatment of SGC7901 cells with fisetin for 48 h resulted in a reduction in the activation of ERK 1/2 in a concentration-dependent manner. The reduction in activation of ERK 1/2 was significant following treatment with 15 μ M fisetin for 48 h. The inhibitory effect of fisetin on activation of ERK 1/2 was further demonstrated using the ERK 1/2 inhibitor, PD98059. The results indicated a significant reduction in the proliferation of SGC7901 cells following treatment with PD98059 ($P < 0.002$). The reduction by PD98059 administration was comparable to that observed following fisetin treatment for 48 h. In conclusion, the current study demonstrates that fisetin inhibits the proliferation of gastric cancer cells and induces apoptosis through suppression of ERK 1/2 activation. Thus, fisetin may have therapeutic applications in the treatment of gastric cancer.

Introduction

Gastric cancer is responsible for ~989,600 incident diagnoses and ~738,000 cases of mortality annually worldwide (1,2). It is detected more commonly in certain regions, including Eastern Asia, Europe and South America (3). Continuous efforts are being made by chemists and clinicians worldwide for improving the prognosis rate of gastric cancer. However, despite the use of various chemotherapeutic agents and surgery, the overall 5-year survival rate of gastric cancer patients is <20% (4,5). Thus, the development of novel and effective treatment strategies for gastric cancer is urgently required.

Natural products isolated from plants, animals, fungi and bacteria exhibit a diverse range of range of biological activities (6-9). They have been used for the treatment of numerous types of disease through the development of innovative drugs (6-9). The main advantage of natural products for the treatment of disease is that they have evolved to possess functionalities that are well-suited as biomolecular frameworks (10). Flavones are the natural products with a wide range of biological activities due to the presence of a benzoquinone pharmacophore (11). The molecule 3,3',4',7-tetrahydroxyflavone, commonly known as fisetin, is a member of the flavonoid family (11). Fisetin is present in fruits and vegetables (12) and its biological evaluation has revealed promising anti-cancer activity. Treatment with fisetin led to inhibition of proliferation and metastasis potential in bladder, pancreatic and cervical carcinoma cells (12,13). In a nude mouse model of prostate cancer, fisetin treatment caused a marked reduction in tumor growth (14). Fisetin has been demonstrated to activate extracellular signal-regulated kinase (ERK) 1/2 in other cell line models (15,16). In the present study, the effect of fisetin on proliferation of gastric carcinoma cells was investigated. The results demonstrate that fisetin treatment inhibits proliferation of gastric carcinoma cells through suppression of ERK 1/2 activation.

Materials and methods

Cell lines and culture. A human gastric cancer cell line, SGC7901, and a normal gastric cell line, GES-1, were obtained from the cell bank of Xiangya Medical School, Central South University (Changsha, China). The cells were cultured in

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Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone; GE Healthcare, Logan, UT, USA), penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 .

Reagents and chemicals. Fisetin and dimethyl sulfoxide (DMSO) were supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The stock solution of fisetin in DMSO was stored at -15°C. The inhibitor for activation of ERK 1/2, PD98059, was obtained from Selleck Chemicals LLC (Shanghai, China).

Analysis of cell proliferation by CCK-8 assay. SGC7901 and GES-1 cell lines were distributed at a density of 2×10^5 cells per well into 96-well culture plates and incubated at 37°C overnight. Then, the medium was replaced with fresh DMEM containing 0 (control), 1, 5, 10, 15 and 20 μM concentrations of fisetin. After 48 h of incubation under a humidified atmosphere of 5% CO_2 at 37°C, 200 μl CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well. The plates were incubated at 37°C for a further 4 h. Then, a microplate reader was used to measure the absorbance at 450 nm. The experiments were performed independently in triplicate.

Analysis of apoptosis using flow cytometry. Apoptosis induction in gastric carcinoma cells following treatment with fisetin was analyzed using an Annexin V/Propidium Iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) apoptosis kit, according to the manufacturer's protocol. SGC-7901 and GES-1 cells were treated with 0 (control), 5, 10 and 15 μM concentrations of fisetin for 48 h under a humidified atmosphere of 5% CO_2 at 37°C. The cells were harvested by trypsinization, washed three times with phosphate-buffered saline (PBS), and resuspended in binding buffer at a concentration of 2×10^7 cells/ml. The cells were then treated with Annexin V-fluorescein isothiocyanate (5 μl) and PI (10 μl) at room temperature in the dark for 10 min. The cells were analyzed using a flow cytometer (FACSAria III; BD Biosciences, Franklin Lakes, NJ, USA). CellQuest software version 3.3 (BD Biosciences) was used for analysis of flow cytometry. The experiments were performed in triplicate for each concentration.

Analysis of cell cycle arrest using flow cytometry. SGC7901 cells at a density of 2.5×10^5 cells/well were distributed into 6-well plates and subjected to incubation for 48 h. RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS was used for cell culture and incubation was performed at 37°C in an atmosphere of 5% CO_2 . The medium was then replaced with fresh medium containing fisetin (15 μM) in DMSO. Following 48 h incubation at 37°C, the cells were subjected to trypsinization and subsequent washing with cold PBS. The cells were then fixed with 70% ethyl alcohol at 4°C for at least 4 h, followed by addition of 20 μl RNase (Thermo Fisher Scientific) and 20 μl PI (Sigma-Aldrich; Merck KGaA). The cells were then incubated for 30 min at 37°C before analysis using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest software version 3.3 (BD Biosciences).

Western blot analysis. The phosphorylation of ERK 1/2 and expression of caspase-7, pro-caspase-7, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bcl-x), BH3 interacting domain death agonist (Bid) and Bcl-2-like protein 11 (Bim) was analyzed using a western blot assay. Effect of PD98059 (ERK 1/2 inhibitor) at 100 μM on activation of ERK 1/2 was also analyzed using this assay. The SGC7901 cells were treated with 15 μM fisetin for 48 h at 37°C under a humidified atmosphere of 5% CO_2 . Following incubation, the cells were treated with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) under ice-cold conditions for 45 min. The cell lysates were subjected to centrifugation at $12,000 \times g$ for 15 min at 4°C. The concentration of proteins in the cell lysates was determined using a bicinchoninic acid assay. The proteins were separated using 10% SDS-PAGE by loading 3 μl protein per lane and subsequently transferred to polyvinylidene difluoride membranes. In the membranes, non-specific sites were blocked with non-fat milk containing Tris-buffered saline with Tween-20. The membranes were incubated with rabbit primary monoclonal antibodies against ERK (cat. no. 137F5; dilution 1:1,000) and p-ERK (cat. no. D13.14.4E; dilution 1:1,000; both from Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C for overnight. The other antibodies used were against Bcl-2 (cat. no. ab7973), Bcl-x (cat. no. ab77566), Bid (cat. no. ab32060), Bim (cat. no. ab32158), β -actin (cat. no. ab8226) and α -tubulin (cat. no. ab7291; all dilution 1:1,000, Abcam, Cambridge, UK). The membranes were washed and incubated with goat anti-rabbit HRP-conjugated polyclonal secondary antibodies (cat. no. 12-348; dilution 1:2,000, Merck KGaA) for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence blotting detection system (FluorChem E; ProteinSimple, San Jose, CA, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation of ≥ 3 experiments performed independently. Statistical analysis was performed with the SPSS 13.0 statistical software (SPSS, Inc. Chicago, IL, USA). A one-way analysis of variance was used, followed by Dunnett's test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Fisetin inhibits proliferation of gastric cancer cells. SGC7901 and GES-1 cells were incubated with various concentrations (1, 5, 10, 15 and 20 μM) of fisetin for 48 h and proliferation was examined. Fisetin treatment at 1, 5, 10, 15 and 20 μM concentration significantly reduced the proliferation rate of SGC7901 cells to 98, 72, 51, 12 and 11%, respectively compared to 100% in control after 48 h ($P < 0.05$; Fig. 1). The proliferation rate of GES-1 cells was found to be 100, 99, 99, 98 and 98% respectively at 1, 5, 10, 15 and 20 μM concentrations of fisetin compared with 100% in untreated cultures (Fig. 1).

Fisetin induces apoptosis in gastric cancer cells. Treatment of SGC7901 cells with various concentrations (5, 10 and 15 μM) of fisetin for 48 h induced cell death in a dose-dependent manner (Fig. 2). Flow cytometry revealed a notable increase in the proportion of apoptotic cells at 15 μM concentration of fisetin after 48 h compared with the untreated control cells

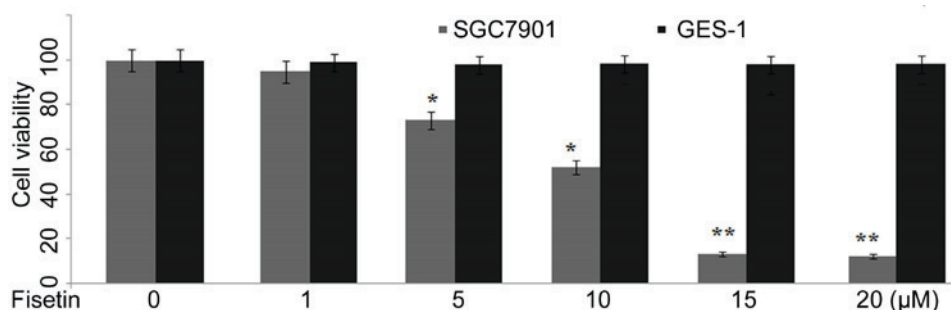


Figure 1. Reduction in the proliferation of SGC7901 gastric cancer cells following fisetin treatment. Cells were treated with 0, 1, 5, 10, 15 or 20 μM fisetin for 48 h. The proliferation of SGC7901 cells was reduced significantly following treatment with fisetin. No effect of fisetin was observed in GES-1 cells. * $P < 0.05$ and ** $P < 0.01$ vs. GES-1 cells.

(Fig. 2). The percentage of apoptotic cells increased to 87% following treatment with 15 μM fisetin compared with 2% in the control for 48 h.

Cell cycle arrest analysis. The effect of fisetin on SGC7901 cell cycle progression was determined using flow cytometry. The results indicated that the percentage of cells in the G2/M and S phases of control cell cultures was 18.23 and 9.14%, respectively (Fig. 3). Following treatment of SGC7901 cells with 15 μM fisetin for 48 h, the proportion of SGC7901 cells at the G2/M and S phases was 23.72 and 8.65%, respectively (Fig. 3). Thus, fisetin treatment increased the proportion of cells at G2/M phase with simultaneous reduction of cells at S phase.

Analysis of caspase-7 and Bcl-2 protein expression. Western blot analysis indicated a marked increase in the expression level of caspase-7 following treatment of SGC7901 cells with 1, 5, 10 and 15 μM fisetin for 48 h (Fig. 4). However, the procaspase-7 expression level was slightly decreased by fisetin treatment (Fig. 4). In SGC7901 cells, fisetin treatment led to a marked decrease in the expression level of anti-apoptotic proteins Bcl-2 and Bcl-x. The expression of Bim was increased and that of Bid decreased following treatment of SGC7901 cells with fisetin for 48 h (Fig. 4).

Inhibition of ERK 1/2 activation in gastric cancer cells by fisetin treatment. Treatment of SGC7901 cells with fisetin for 48 h resulted in marked reduction of the activation of ERK 1/2 in a concentration-dependent manner (Fig. 5).

The inhibitory effect of fisetin on activation of ERK 1/2 was further demonstrated using an ERK 1/2 inhibitor, PD98059. The results indicated a marked decrease in the proliferation of SGC7901 cells compared with control cells, following treatment with 100 μM PD98059 ($P < 0.002$). The reduction by PD98059 administration was similar to that observed following fisetin (15 μM) treatment for 48 h (Fig. 6). Furthermore, PD98059 was observed to markedly reduce the activation of ERK 1/2 in SGC7901 cells (Fig. 7). Inhibition of ERK 1/2 activation by PD98059 produced similar results to fisetin treatment (15 μM) for 48 h (Fig. 7).

Discussion

The current study demonstrates the effect of fisetin on the proliferation of gastric cancer cells and provides insight into

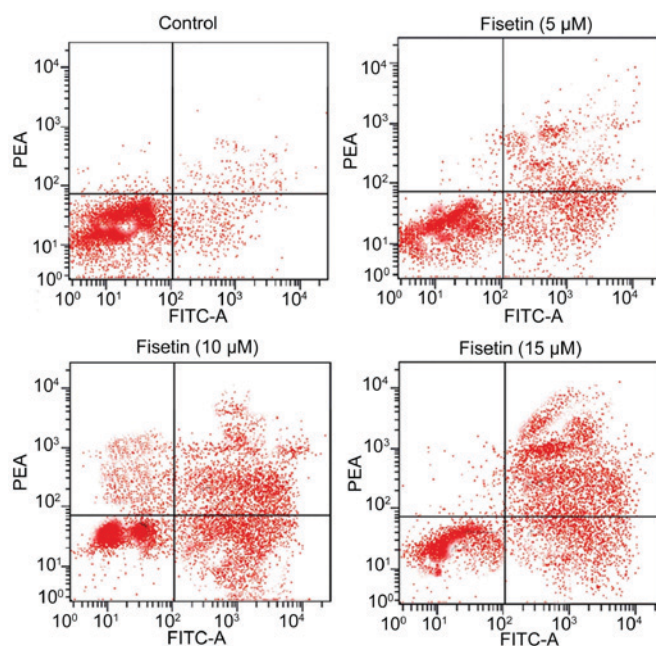


Figure 2. Fisetin treatment induces apoptosis in SGC7901 cells. Cells were treated with 5, 10 and 15 μM fisetin, or left untreated (control; 0 μM) for 48 h and then analyzed using flow cytometry. A significant increase in the apoptotic cell population was observed following treatment with fisetin. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

its underlying mechanism. The results demonstrated that fisetin treatment inhibited proliferation and induced apoptosis in SGC7901 cells via inhibition of ERK 1/2 activation.

Fisetin treatment has been identified to inhibit the proliferation and metastasis potential of numerous types of carcinoma cell, including bladder, pancreas and cervical carcinoma (11-13). In the current study, fisetin treatment (10-20 μM) of SGC7901 cells under acidic conditions led to a significant due to the generation of acidic by-products during the process of glycolysis (17,18). The rate of proliferation is inhibited in gastric cancer cells through induction of apoptosis (19). The current study revealed that treatment of SGC7901 cells with fisetin for 48 h induced apoptosis in a dose-dependent manner. The percentage of apoptotic cells increased to 87% following treatment with 15 μM fisetin for 48 h. The inducers of apoptosis include caspase-3 and -7, since their expression causes morphological changes in cells that are characteristic of apoptosis (20).

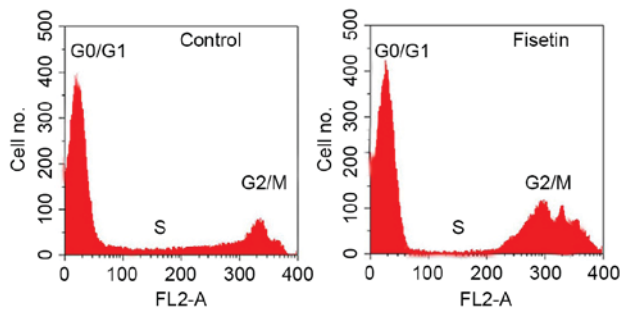


Figure 3. Cell cycle distribution of untreated control SGC7901 cells and SGC7901 cells treated with 15 μ M fisetin for 48 h. Cell cycle distribution was analyzed using flow cytometry.

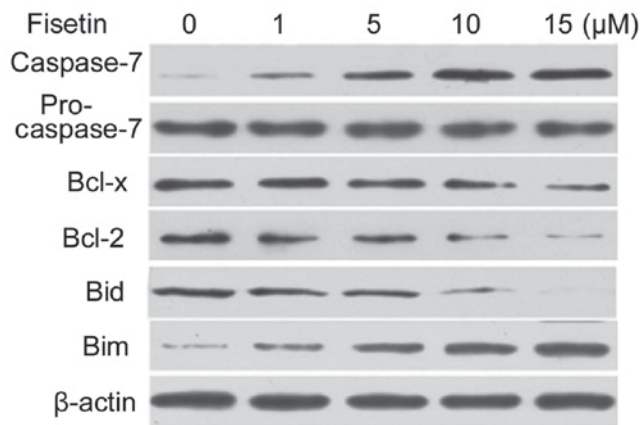


Figure 4. Western blot analysis of caspase-7, procaspase-7, Bcl-2, Bcl-x, Bid and Bim expression in SGC7901 cells treated with 0, 1, 5, 10 or 15 μ M fisetin for 48 h. β -actin was used as an internal control.

The current results suggested that fisetin treatment of SGC7901 cells caused apoptosis induction by activating caspase-7 and reducing the expression of anti-apoptotic proteins Bcl-2, Bcl-x and Bid. The expression of pro-apoptotic protein Bim was increased following treatment of cells with fisetin.

It has been reported that the rate of proliferation is inhibited in gastric cancer cells through induction of apoptosis via targeting the mitogen-activated protein kinase (MAPK) pathway (21). It was reported that ERK1/2 phosphorylation can be selectively inhibited either by its inhibitor (PD98059) or by using drugs, including matrine (22). The proliferation of cancer cells is regulated by one of the important members of the MAPK family, ERK 1/2 (23). The current results revealed that treatment of SGC7901 cells with fisetin for 48 h led to a reduction in the activation of ERK 1/2 in a concentration-dependent manner. The inhibitory effect of fisetin on activation of ERK 1/2 was further demonstrated using ERK 1/2 inhibitor, PD98059. The results indicated a significant reduction in the proliferation of SGC7901 cells following treatment with PD98059. The reduction by PD98059 administration was comparable to that observed following fisetin treatment for 48 h. PD98059 treatment was also observed to markedly reduce the activation of ERK 1/2 in SGC7901 cells. This inhibition was similar to the result observed for fisetin treatment (15 μ M).

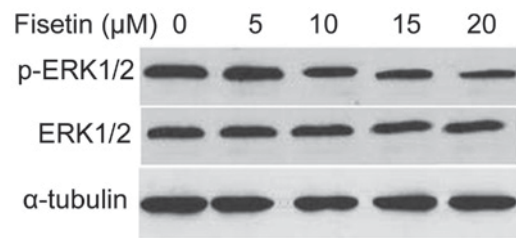


Figure 5. Fisetin treatment inhibits activation of ERK 1/2 in SGC7901 cells. The cells were treated with various concentrations (0, 5, 10, 15 and 20 μ M) of fisetin for 48 h, then subjected to western blotting. The activation of ERK 1/2 in fisetin-treated SGC7901 cells was markedly lower compared with the untreated control cells. ERK, extracellular signal-regulated kinase.

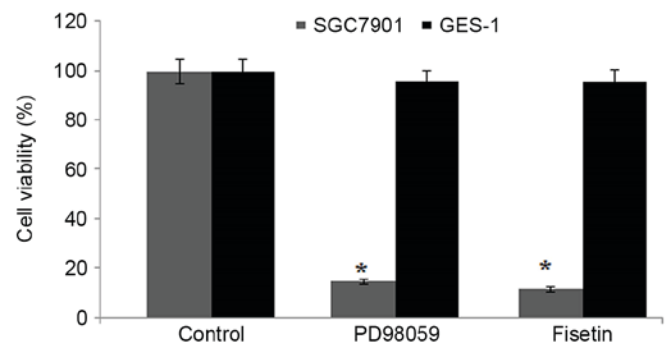


Figure 6. Effect of ERK 1/2 inhibitor on the proliferation of SGC7901 cells. Cells were treated for 48 h with PD98059 (100 μ M) or fisetin (15 μ M) and then analyzed for proliferation. PD98059 and fisetin significantly inhibited the proliferation of SGC7901 cells compared to control cells. * P <0.002 vs. control SGC7901 cells. ERK, extracellular signal-regulated kinase.

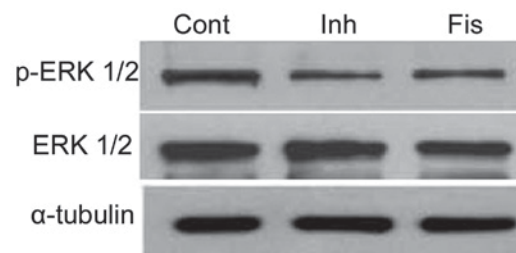


Figure 7. Effect of ERK 1/2 inhibitor on the activation of ERK 1/2 in SGC7901 cells. Cells were treated for 5 h with PD98059 (100 μ M) or for 48 h with fisetin (15 μ M) and then analyzed using western blotting for the activation of ERK 1/2. PD98059 and fisetin were observed to inhibit the activation of ERK 1/2 in SGC7901 cells. ERK, extracellular signal-regulated kinase.

In conclusion, the current study demonstrates that fisetin inhibits the proliferation of gastric cancer cells and induces apoptosis through suppression of ERK 1/2 activation. Thus, fisetin may have therapeutic applications in the treatment of gastric cancer.

Competing interests

The authors declare that they have no competing interests.

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