Fisetin Reduces Cell Viability Through Up-Regulation of Phosphorylation of ERK1/2 in Cholangiocarcinoma Cells

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Abstract. *Background:* Cholangiocarcinoma (CCA) is a malignancy with poor prognosis and limited therapeutic options. Effective prevention and treatment of CCA require developing novel anticancer agents and improved therapeutic regimens. As natural products are considered a rich source of potential anticancer agents, we investigated the anticancer effect of fisetin in combination with gemcitabine. *Materials and Methods:* Cytotoxic effect of fisetin and gemcitabine on a human CCA cell line SNU-308 was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and apoptosis assay using propidium iodine and annexin V. *Molecular mechanisms of fisetin action in CCA were investigated by western blotting. Results:* Fisetin was found to inhibit survival of CCA cells, through strongly phosphorylating ERK. It also induced cellular apoptosis additively in combination with gemcitabine. Expression of cellular proliferative markers, such as phospho-p65 and myelocytomatosis (MYC), were reduced by fisetin. *Conclusion:* These results suggest fisetin in combination with gemcitabine as a candidate for use in improved anticancer regimens.

Cholangiocarcinoma (CCA) is the second most common primary hepatic malignancy worldwide (1, 2). Surgical resection is the only effective therapeutic option for CCA, but this is applicable in fewer than 50% of cases because this

type of cancer is mostly diagnosed at a late stage. Currently, there are no curative medical therapies for CCA. Targeted therapies have failed or shown only marginal benefits in several clinical trials with different drugs alone or in combination with chemotherapy (3-5). The development of effective targeted therapies in CCA is challenging because of the underlying genetic variability of the disease and the remarkable resistance of CCA cells to drug cytotoxicity (6). Systemic chemotherapy is increasingly being applied in cases of advanced CCA. One combination treatment is gemcitabine with cisplatin (7). The median overall survival with cisplatin plus gemcitabine was found to be significantly greater compared with that using gemcitabine alone (11.7 versus 8.1 months), however, it is not curable only palliative (7). Furthermore, combination treatment contributes to added toxicity and strong drug resistance (8-11).

Novel strategies for sensitizing tumor cells with naturally-occurring dietary chemopreventive compounds have gained considerable attention because of their beneficial effects in overcoming tumor cell resistance to apoptosis (12). Soy isoflavones including genistein, daidzein, and glycitein, mainly derived from soybeans, have been found to inhibit breast cancer growth *in vivo* and *in vitro* (13). Indole-3-carbinol and its *in vivo* dimeric product diindolylmethane, produced from naturally occurring glucosinolates in the *Cruciferae* family, have been shown to inhibit prostate cancer cell growth through the modulation of genes related to the control of cell proliferation, cell cycle, apoptosis, signal transduction, oncogenesis, and transcription regulation (14). One flavonoid, apigenin, is widely distributed in many fruits and vegetables, and has been shown to exert anti-inflammatory and anticancer effects alone and in combination with gemcitabine (15, 16). Guggulsterone (4,18[20]-pregnadiene-3,16-dione), a polyphenol from *Commiphora mukul.*, also increased apoptosis of pancreatic cancer cells in combination with gemcitabine (17).

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Fisetin (3,3',4',7-tetrahydroxyflavone) is a bioactive flavone present in various fruits and vegetables, including strawberry, grape, apple, onion, and cucumber (18). Dietary fisetin is believed to have a number of health-promoting effects that include antioxidant, anticarcinogenic, antiproliferative and apoptotic activities (19). Fisetin is cytotoxic towards non-small cell lung (20), colon (21-22), prostate (23) and pancreatic (24) cancer cells, as well as malignant melanoma cells (25). Fisetin causes cell-cycle arrest through the inhibition of Aurora B kinase directly in several cancer types (26). In addition, fisetin down-regulates nuclear factor κ B (NF- κ B) *via* mitogen-activated protein kinase signaling pathways during cancer metastasis (27, 28).

Rat sarcoma (RAS)–extracellular signal-regulated kinase (ERK) (29) and phosphoinositide 3-kinase (PI3K)–AKR mouse thymoma kinase (AKT) (30) signaling pathways play central roles in multiple processes associated with cancer. The RAS–ERK signaling axis stimulates cell proliferation and the PI3K–AKT signaling axis promotes cell survival. Genetic alterations in critical components in these pathways result in unrestricted cellular proliferation and reduced sensitivity to chemotherapies in cancer. Controlling these pathways using inhibitors is a key therapeutic target for cancer therapy.

In this study, we investigated the effect of fisetin on a human CCA cell line, SNU-308. We also explored the action mechanisms of fisetin on CCA and of the potential combination treatment using fisetin and gemcitabine.

Materials and Methods

Cell culture and reagents. SNU-308, a human CCA cell line, was obtained from the Korean Cell Line Bank (Seoul, Korea). SNU-308 cells were grown in Roswell Park Memorial Institute 1640 (RPMI-1640; Welgene, Gyeongsan, Korea) with 1% streptomycin and penicillin (Corning, Corning, NY, USA), 5 mM sodium pyruvate (PAN BIOTECH, Aidenbach, Germany) and 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA, USA). The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment. For experiments, cells were seeded in 60 mm culture dishes (4×10⁵ cells/dish), and allowed to attach overnight. Compounds (200 μ M fisetin with/without 150 μ M gemcitabine) were added, and the cells were incubated for 16, 24, 48 or 72 h. Cells were treated with Dimethyl sulfoxide (DMSO) (0.25%) as vehicle control.

3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded on 96-well plates containing a final volume of 100 μ l/well at a density of 4×10³ cells/well, incubated at 37°C for 24 h and then treated with 200 μ M fisetin alone and with 150 μ M gemcitabine for 24, 48, and 72 h. In order to determine the live cell numbers, 10 μ l of 5 mg/ml MTT (Promega, Madison, WI, USA) in phosphate-buffered saline (PBS) was added to the cells and allowed to develop for 1 h at 37°C. To dissolve formazan crystals, 100 μ l of 10% sodium dodecyl sulfate (SDS) solution was added to cells and they were incubated overnight at room temperature (RT).

The solution was mixed to ensure complete solubilization. Colorimetric measurements were taken at 570 nm by a Sunrise™ reader (Tecan, Mannedorf, Switzerland).

Apoptosis assay. Apoptosis was examined using a double-staining method with allophycocyanin (APC)-labeled annexin-V (AV) and propidium iodide (PI) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). For PI and AV staining, cells (1×10⁵) were suspended with 100 μ l of Annexin-binding buffer (BD Biosciences). Cell suspension was stained with 5 μ l of APC-conjugated AV and 5 μ l of PI for 15 min at RT and then 400 μ l of annexin-binding buffer was added. Apoptotic and necrotic cells were analyzed with a flow cytometer (FACScanto II; BD Biosciences). Data analysis was performed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

Protein extraction and western blot analysis. Cells were harvested after treatment of fisetin with/without gemcitabine for 16 h, washed twice in PBS and then suspended in 20 μ l of lysis buffer (50 mM Tris-HCl at pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, Protease Inhibitor cocktails (Roche, Basel, Switzerland), 5 mM NaF, 2 mM Na₃VO₄). Cell suspensions were kept on ice for 15 min and then centrifuged at 13,500 × g at 4°C for 15 min. Protein concentrations were determined by the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturers' instructions. An equal amount (30 μ g) of each protein sample was loaded into each lane, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gradient, and then transferred onto polyvinylidene difluoride membrane (Amersham Biosciences, Amersham, UK). Membranes were blocked with 3% bovine serum albumin in PBS containing 1% Tween-20 (PBS-T) for 30 min at RT and followed by incubation with primary antibodies overnight at 4°. Membranes were washed three times in PBS-T and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibody. Bands were visualized using an enhanced chemiluminescence detection system (ImageQuant LAS 4000; GE Healthcare, Uppsala, Sweden). Antibodies against phospho-AKT (monoclonal), AKT (polyclonal), phospho-ERK (monoclonal), ERK (monoclonal), and beta-actin (monoclonal) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). horseradish peroxidase-conjugated secondary antibody, phospho-p65 (monoclonal) and p65 (polyclonal) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Quantification of band intensity was assessed with Image Studio™ Lite Ver. 5.2 (Li-COR Bioscience, Lincoln, NE, USA).

Quantification of mRNA of MYC. Total RNA was extracted using TRIzol™ (Thermo Fisher Scientific) reagent following the manufacturer's instructions. The quantity and purity of total RNA were measured using an UV/Vis-Spectrophotometer (NanoDrop ND-2000; Thermo Fisher Scientific). cDNA was synthesized with 2 μ g of total RNA using the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo d(T) primers (Invitrogen). The amplification was performed in 20 μ l, containing 1 μ l of each primer of 5 pM. SYBR Green PCR master mix (Applied Biosystems) and 2 μ l of 1:10 diluted cDNA. Amplification conditions were 95°C for 10 s, and 60°C for 1 min for 40 cycles. Primer sequences were as follows: MYC forward: 5'-AAA GGC CCC CAA GGT AGT TA-3', and reverse: 5'-TTT CCG CAA CAA GTC CTC TT-3' (31). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts

were amplified as a reference standard. *GAPDH* primers were as follows: forward: 5'-ACC CAG AAG ACT GTG GAT GG-3', and reverse: 5'-TTC AGC TCA GGG ATG ACC TT-3'. All primers were synthesized by Bioneer Co. (Daejeon, Korea).

Statistical methods. Statistical comparisons were made using the two-tailed Students' *t*-test using Excel software (Microsoft, Seattle, WA, USA). Results were considered significant in all experiments at $p < 0.05$. Data are expressed as the mean $\pm$ SEM.

Results

Fisetin reduces cell viability and induces apoptosis in combination with gemcitabine. The effect of fisetin alone and in combination with gemcitabine on survival and proliferation of SNU-308 cells was examined by the MTT assay at different incubation times (24, 48 and 72 h). The concentration of each agent was selected to produce moderate cell death, as we aimed to investigate additive or synergistic effects of both agents. The combinatory treatment of fisetin and gemcitabine reduced cell viability compared to a single treatments (Figure 1A). Survival of the cells treated with fisetin and gemcitabine in combination was reduced by 34.2% at 72 h compared to vehicle control, which was treated with DMSO. We tested the effect of fisetin on two other CCA cell lines (SNU-1079 and SNU1196) as well, and found that fisetin indeed reduced survival of both cell lines, but susceptibility depended on the cell line (data not shown).

To confirm apoptotic induction by fisetin and gemcitabine, cells were labeled with AV and PI. Fisetin alone induced apoptosis (PI⁻/AV⁺ early apoptotic cells and PI⁺/AV⁺ late apoptotic/necrotic cells). Fisetin and gemcitabine used alone increased the proportion of early apoptotic cells (Figure 1B and C). Exposure to fisetin (200 μ M) with gemcitabine (150 μ M) further induced apoptosis, in up to 62.4% of SNU-308 cells, as seen in the representative result shown in Figure 1B. The combination treatment of fisetin and gemcitabine induced apoptosis more efficiently than gemcitabine alone (29.2%). Taken together, fisetin reduced SNU-308 cell numbers by inducing apoptosis.

Fisetin induces activation of ERK1/2 and AKT. In order to determine which signaling pathways were affected by fisetin, immunoblotting was performed to detect ERK, phospho-ERK, AKT and phospho-AKT. In addition, immunoblotting for p65 and phospho-p65 was performed to test whether fisetin affected the NF- κ B pathway in SNU-308 cells. Interestingly, fisetin alone strongly induced phosphorylation of ERK1/2, and phospho-AKT appeared to increase by fisetin, although the difference was not significant (Figure 2). Fisetin reduced slightly, if at all, phosphorylation of p65, a subunit of NF- κ B complex. Taken together, upregulation of ERK activation in RAS-ERK pathway by fisetin was most outstanding and might be the cause of reduced CCA cell

viability. However, gemcitabine alone and combination of fisetin and gemcitabine did not further increase phosphorylation of AKT and ERK. Only NF- κ B activation was increased by combinatory treatment. In conclusion, the effect of fisetin and gemcitabine on SNU-308 cell viability did not seem to be the result of additive stimulation of the same signaling pathways, and fisetin and gemcitabine may have distinct action mechanisms.

Fisetin down-regulates the expression of c-MYC at the transcription level. MYC, a transcription factor, is an important downstream target of RAS-ERK signaling. In order to address whether increased phosphorylation of ERK1/2 affected MYC expression, the mRNA level of *MYC* was assessed by quantitative reverse transcription polymerase chain reaction. The results from fisetin-treated cells showed 5-fold decrease in the mRNA level of the *MYC* gene (Figure 3), suggesting that fisetin down-regulated *MYC* expression not stimulated it. However, it should be mentioned that co-treatment of fisetin and gemcitabine did not affect *MYC* expression, as it did not affect phosphorylation of ERK. This result implies that cellular proliferation, which *MYC* promotes, might be blocked by fisetin.

Discussion

CCA is characterized by a poor prognosis, with a median survival of less than 24 months (32) and a limited response to chemotherapy. The number of clinical trials with targeted therapy alone and in combination with traditional chemotherapy is expanding for advanced-stage CCA. However, the major problem is due to treatment-associated toxicity with no significant added benefit in overall survival (7). Moreover, CCA is characterized by a remarkable resistance to common chemotherapy (33, 34). In order to overcome these limitations of current chemotherapeutic regimens, we evaluated the efficacy of fisetin, a nontoxic flavonoid compound, in combination with a common chemotherapeutic agent, gemcitabine, against SNU-308 CCA cells. This study documents that sensitization of cancer cells was achieved by fisetin during gemcitabine-induced killing, as shown by more pronounced cell death compared with single-agent treatment.

It is widely accepted that chemotherapy acts primarily through blocking the survival and proliferation signaling pathways of cancer cells, and that alteration in these signaling pathways can make cancer cells resistant to therapy (35). In particular, RAS-ERK and PI3K-AKT signaling pathways contribute to cancer proliferation and cell survival, respectively (35). In normal cells, these pathways are transiently activated in response to growth factors, cytokines and ligands of integrin, but genetic alterations can lead to constitutive signaling even in the absence of growth factors.

Figure 1. Fisetin (Fis) inhibits cell proliferation and induces apoptosis in combination with gemcitabine (Gem). A: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that fisetin with/without gemcitabine inhibited the growth of SNU-308 cells in a time-dependent manner. The experiments were performed in triplicates and the graph is representative of three independent experiments. Data represent the means $\pm$ SEM of triplicates. * $p < 0.05$ between the fisetin-treated cells and the dimethyl sulfoxide (DMSO)-treated cells at 48 h by Student's *t*-test. B: SNU-308 cells were stained with allophycocyanin-conjugated annexin V and propidium iodide (PI) and analyzed by flow cytometry after fisetin/gemcitabine treatment for 16 h. C: Percentage of early apoptotic cells (PI-/annexin V⁺) and late apoptotic and necrotic cells (PI+/annexin V⁺) are displayed in the graphs. Data represent the means $\pm$ SEM of three independent experiments.

However, the role of ERK is controversial, considering that its activation can result in either cell proliferation or cell death. It is reported that the activation of ERK can be a result of DNA damage that subsequently leads to cell-cycle

arrest and apoptosis (36, 37). In addition, ERK activation has been suggested to be a response to counteract endoplasmic reticulum stress-induced apoptosis (38, 39), induced by external stimuli such as cytotoxic agents.

Figure 2. Fisetin (Fis)-induced apoptosis mediated by phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). A: The protein levels of phospho-ERK, ERK, phospho- v-akt murine thymoma viral oncogene homolog (AKT), AKT, phospho-p65, p65 and β -actin were examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis after 16 h of designated drug treatment. The results are representative of three independent experiments. B: Relative intensities of bands of phosphorylated proteins to total proteins are displayed in the graphs. Data represent the means $\pm$ SEM of three independent experiments. Gem: Gemcitabine.

Figure 3. Fisetin (Fis) down-regulates the expression of myelocytomatosis (MYC) gene at the transcription level. Quantitative reverse transcription polymerase chain reaction was performed to assess MYC mRNA level in SNU-308 cells treated with fisetin with/without gemcitabine (Gem) for 16 h. Data represent the means $\pm$ SEM of three independent experiments performed in triplicates.

In this study, we found that fisetin induced strong phosphorylation of ERK1/2 and down-regulated MYC. The strong or sustained activation of mitogen-activated protein kinase pathways arrests the cell cycle, whereas transient activation induces cell-cycle progression (40). In line with these results, low levels of v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) activity induce cyclin D1 and therefore proliferation, whereas high levels lead to *p21* induction and growth arrest (41, 42). High levels of ERK1/2 activity as a result of fisetin treatment leads to MYC repression and therefore *p21* induction (21). The hypothetical mechanism of action of fisetin is depicted in Figure 4. Fisetin seems to lead to arrest of hypermitogenic cell cycle, increasing susceptibility to gemcitabine. Gemcitabine is an intravenous drug that is metabolized within tumor cells and induces a programmed cell death response by blocking the progression of dividing cells through the G₁/S phase boundary (43). In general, gemcitabine treatment results in the accumulation of the phosphorylated forms of checkpoint kinase 1 (CHK1) and CHK2 and degradation of cell division cycle 25A (43-45). It seems that gemcitabine-induced CHK1 activation functions in part to coordinate cell-cycle progression with DNA synthesis, preventing cells with stalled replication from prematurely entering mitosis (43). Thus, gemcitabine-treated cells are arrested in the early S-phase (43). It is likely that gemcitabine shifts cell-cycle arrest by fisetin via

Figure 4. Schematic view of hypothetical model of action of fisetin showing that fisetin induces apoptosis of SNU-308 cholangiocarcinoma cells through up-regulation of phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog (RAS)– extracellular signal-regulated kinase (ERK) signaling pathway, and reduced myelocytomatosis (MYC) expression also contributes to reduced proliferation of cholangiocarcinoma (CCA) cells. MEK1/2: Mitogen-activated protein kinase 1/2; RAF: v-raf-1 murine leukemia viral oncogene homolog; CDK: cell cycle-dependent kinase.

ERK-dependent signaling pathway to apoptosis irreversibly via an alternative signaling pathway. It is possible that dual treatment of fisetin and gemcitabine might induce apoptosis and inhibit cell proliferation, respectively, leading to an additive anticancer effect.

In conclusion, we demonstrated that fisetin seems to induce cell death through the RAS–ERK pathway and sensitization of cancer cells achieved by fisetin induces cell

death through an alternative pathway by gemcitabine. These results imply that alternative pathways that control cell proliferation and survival might be prevented by combination treatment with fisetin and gemcitabine, making them attractive agents for CCA treatment.

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