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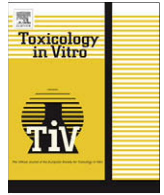

Brief communication

# 2′,4′-Dihydroxychalcone-induced apoptosis of human gastric cancer MGC-803 cells via down-regulation of survivin mRNA

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ABSTRACT

2′,4′-Dihydroxychalcone (TFC), a main component in *Herba Oxytropis*, is grouped under flavonoids, which are well known to have antitumor activities in vitro. In this study, the possible antitumor mechanism of TFC in human gastric cancer MGC-803 cells is examined. Hoechst 33258 staining analysis indicates that TFC causes MGC-803 cell shrinkage and apoptotic body formation, typical characteristics of apoptosis. Flow cytometric analysis demonstrates that TFC causes cell cycle arrest in the G2/M phase. Furthermore, TFC significantly increases caspase-3 activity but decreases survivin mRNA expression. Therefore, TFC can induce the apoptosis of MGC-803 cells via down-regulation of survivin mRNA expression.

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## 1. Introduction

*Herba Oxytropis*, named “Er Da Ga” in Tibetan, is a wild-growing Leguminosae plant mainly distributed over the Qinghai-Tibet Plateau in China. The herb of this plant is known as one of the “Three Anti-inflammatory Drugs” and the “King of Herb” in Chinese Tibetan medicine, and is used to treat leprosy, trauma, influenza, and tonsillitis for thousands of years (Mao et al., 1986; Jiang et al., 2006; Luo, 1997). At present, many Chinese traditional patent prescriptions containing this medicine abound in the market. Alcohol extracts of *Herba Oxytropis* mixed with other medicinal materials (Qingpenggao) have been patented as a treatment for pain and arthritis (Lei et al., 2008). However, little information exists regarding the pharmacologic effects of this herb (Wei et al., 1979; Wang et al., 2008; Tong et al., 2008).

2′,4′-Dihydroxychalcone (TFC) is one of the main components in *Herba Oxytropis*. Experimental studies have shown the antiproliferative activity of TFC on cancer cells, including uterus, ovary, lung, colon, prostate, and breast tumor cell lines (Pouget et al., 2001; Chen et al., 2005; Iwata et al., 1995; Kachadourian and Day, 2006). Although there are some reports about the antitumor mechanism of chalcone (Hsu et al., 2006; Liu et al., 2007), there is still limited information about the antitumor mechanism of TFC.

Survivin, a member of the inhibitor of apoptosis family, inhibits the activation of downstream effectors of apoptosis (i.e., caspase-3 and -7) in cells exposed to apoptotic stimuli (Shin et al., 2001). Elevated survivin expression is present in fetal tissues and in human cancers of various origins, such as breast, lung, prostate, colon, pancreas, and stomach (Ambrosini et al., 1997; Lu et al., 1998; Tanaka et al., 2000; Kawasaki et al., 1998; Monzo et al., 1999). In this study, we investigate the effect of TFC on the expression of survivin mRNA in gastric cancer cells. Results of this investigation may provide a scientific explanation for the antitumor mechanism of TFC.

## 2. Materials and methods

### 2.1. Materials

TFC was isolated from the *Oxytropis falcata* herb in Jiangsu Key Laboratory of Chinese Medicine Processing as described by Yang et al. (2008). Previous experiments showed that TFC purity was above 95% (confirmed by high-performance liquid chromatography and spectral analysis). The TFC structure is shown in Fig. 1. The compound was dissolved in dimethylsulfoxide (DMSO). Control cells were treated with the same amount of vehicle alone. Final DMSO concentration never exceeded 1% (v/v) in both control and treated samples. Reverse Transcription System was purchased

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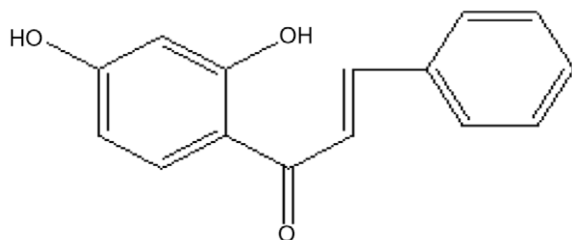


**Fig. 1.** Structure of 2′,4′-dihydroxychalcone.

from Promega (Madison, WI, USA); Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY, USA); fetal bovine serum was purchased from Hangzhou Sijiqing Co., Ltd. (Hangzhou, China); and propidium iodide (PI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were of analytical grade from commercial suppliers.

### 2.2. Cell culture

Human gastric cancer cell line (MGC-803) was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China) and maintained in DMEM plus 10% calf serum, 100 U/ml penicillin, and 75 U/ml streptomycin in a 37 °C incubator supplied with 95% room air and 5% $CO_2$. After 60–80% confluency, cells were trypsinized with 0.25% trypsin (AMRESCO, dissolved in phosphate-buffered saline (PBS), pH 7.4), counted, and placed down at a needed density for treatment.

### 2.3. Hoechst 33258 staining for apoptotic cells

Hoechst 33258 staining was carried out as previously described (Ramonede and Tomas, 2002). Briefly, MGC-803 cells in exponential growth were placed down at a final concentration of $5 \times 10^5$ cells per well in a six-well plate. A coverslip was placed on the bottom of each well to allow cells to grow on its surface as a monolayer. After 24 h, cells were treated with 0, 5, 10, and 20 μg/ml TFC for 48 h. Subsequently, cells were fixed, washed twice with PBS, and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Haimen, China). Changes in the nuclei of cells after Hoechst 33258 staining were observed under a fluorescence microscope (Olympus, BX-60, Japan) in less than 15 min.

### 2.4. Assessment of cell cycle distribution

The cell cycle of MGC-803 cells was determined by flow cytometry based on a previously described method (Yin et al., 2003). Briefly, MGC-803 cells were placed down at a density of $1 \times 10^6$ cells per well in a six-well plate. After treatment, adherent and nonadherent cells were collected, washed twice with ice-cold PBS (pH 7.4), fixed with 70% alcohol overnight, and stained with PI (1 mg/ml) in the presence of 1% RNase A for at least 30 min before analysis by flow cytometry (Becton Dickinson, USA). G0/G1, S, and G2/M cells were gated out as appropriate. Data were analyzed with Modfit and CellQuest software.

### 2.5. Caspase-3 activity assay

Caspase-3 activity was determined using the Caspase-3 Activity Kit (Beyotime, Haimen, China). According to the manufacturer's protocol, treated cells were lysed with lysis buffer (100 μl per $2 \times 10^6$ cells) for 15 min on ice followed by washing with cold PBS. After incubation of the mixture consisting of 10 μl cell lysate, 80 μl reaction buffer, and 10 μl 2 mM caspase-3 substrate (Ac-DEVDpNA) in 96-well microtiter plates at 37 °C overnight, caspase-3 activity in the samples was quantified with a microplate spectrophotometer at 405 nm. Caspase-3 activity was expressed as the fold of enzyme activity compared to the control.

### 2.6. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of survivin expression in MGC-803 cells

RT-PCR analysis of survivin expression in MGC-803 cells was carried out as previously described (Ryan et al., 2005). Briefly, total RNA was isolated by Trizol reagent and cDNA was synthesized from 1 μg total RNA. The primer sequences were 5′-GCATGGG TGCCCCGACGTTG-3′ and 5′-GCTCCGGCCAGAGGCCTCAA-3′ for survivin and 5′-GCCTCAAGATCATCAGCAA-3′ and 5′-CCAGCGTCAAAG GTGGAG-3′ for glyceraldehyde phosphate dehydrogenase (GAPDH) used as an internal control. Amplification conditions were as follows: 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 1 min at 62 °C, and 1 min at 72 °C with a final extension of 5 min at 72 °C. PCR products were visualized on a 1.5% agarose gel. Gels were stained with ethidium bromide, destained, and photographed by the image analysis system. Each band of the electrophoresis gel was then semiquantitatively analyzed using Imagemaster and Software Image Analyzer (Jieda, Suzhou, China). Intensities of the mRNA levels were normalized to those of the GAPDH products as ratios to produce arbitrary units of relative abundance.

### 2.7. Statistical analysis

Results were expressed as mean ± S.D. Statistical comparison between experimental group and control was performed using unpaired two-tailed Student's *t*-test. The criterion of statistical significance was taken as $^*P < 0.05$.

## 3. Results

### 3.1. Effects of TFC on nuclear morphology

To determine whether the growth-inhibitory effect of TFC is related to the induction of apoptosis, treated cells were analyzed using Hoechst 33258 staining by fluorescence microscopy. As shown in Fig. 2a, the control is morphologically normal. The nuclei of different cells are similarly sized, regularly shaped, and evenly stained. With TFC treatment, however, cells show marked morphological changes. In groups treated with 5 and 10 μg/ml TFC (Fig. 2b and c, respectively), cells reveal marked nuclear condensation, nuclear fragmentation, and apoptotic bodies, all of which are characteristics of apoptosis. However, in groups treated with 20 μg/ml TFC (Fig. 2d), most MGC-803 cells detach from the surface of the coverslips and float in the medium. The nuclear morphology of cells on the coverslips is completely destroyed.

### 3.2. Effects of TFC on cell cycle

Flow cytometry was used to detect cell cycle in TFC-treated cells. Results (Table 1) show that TFC (0–10 μg/ml) results in a dose-dependent G2/M phase cell cycle arrest of MGC-803 cells. The percentage of cells in the G2/M phase increases from 18.36% (without TFC) to 60.53% (treated with 10 μg/ml TFC). However, the effects decrease after treatment with 20 μg/ml TFC (the percentage of cells in G2/M phase is 30.60%). The percentage of cells in S-phase increases after treatment with 5 μg/ml TFC (44.72% versus 30.51% in control).

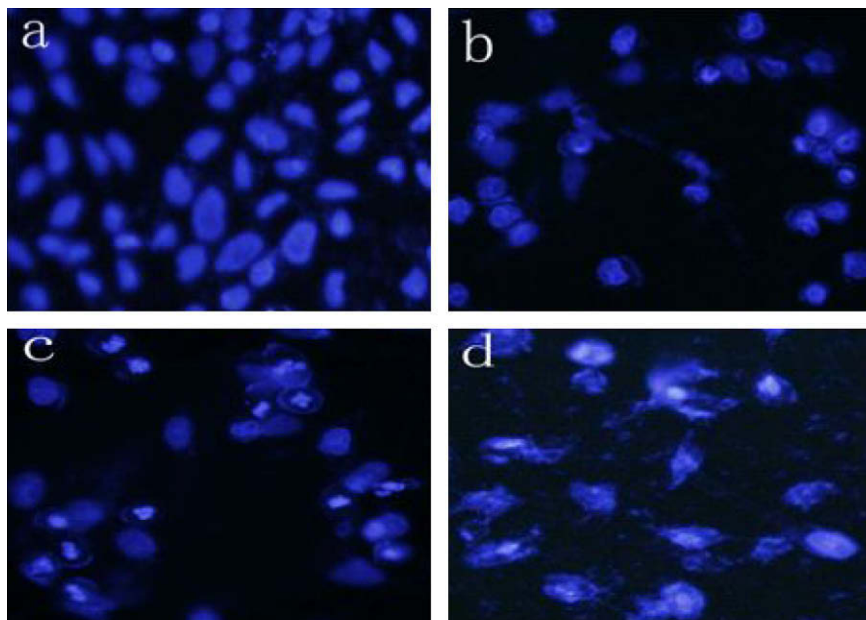


**Fig. 2.** Effects of TFC on the nuclear morphology of MGC-803 cells. Fluorescence photomicrographs of MGC-803 cells stained with Hoechst 33258 after treatment with TFC (×40). Cells were treated with or without TFC for 48 h. Then, cells were stained with Hoechst 33258 and morphology was immediately assessed using fluorescence microscopy: (a) control cells without TFC, (b) TFC 5 μg/ml, (c) TFC 10 μg/ml, and (d) TFC 20 μg/ml. With TFC treatment, cells show marked morphological changes.

**Table 1**
Cell cycle distribution of MGC-803 after treatment with TFC for 48 h.

| TFC (μg/ml) | Cell cycle distribution of MGC-803 (%) | | |
|---|---|---|---|
| | G0/G1 | G2/M | S |
| 0 | 51.13 | 18.36 | 30.51 |
| 5 | 28.57 | 26.71 | 44.72 |
| 10 | 30.55 | 60.53 | 8.92 |
| 20 | 45.50 | 30.60 | 23.90 |

G0/G1, S, and G2/M cells were gated out as appropriate. Data were analyzed with Modfit and CellQuest software.

### 3.3. Effects of TFC on caspase-3 activity

Caspase-3 is the final executor of apoptotic DNA damage, and its activity is a characteristic of apoptosis. Treatment with TFC (2.5–20 μg/ml) for 48 h leads to caspase-3 activities at 106.17 ± 6.41, 108.90 ± 3.72, 112.34 ± 3.40, and 117.11 ± 3.66 compared with those of the control (without TFC) (Fig. 3). Caspase-3 activities increase ($P < 0.05$) when TFC concentration is at 5, 10, and 20 μg/ml. Thus, TFC increases caspase-3 activity in MGC-803 cells in a dose-dependent manner.

### 3.4. Survivin mRNA expression of MGC-803 cells treated with TFC by RT-PCR

RT-PCR analysis demonstrates that survivin mRNA expression is down-regulated by TFC treatments (Fig. 4). A decrease in survivin mRNA is observed with increasing TFC concentration. Survivin mRNA expression is down-regulated ($P < 0.05$) when TFC concentration is at 10 and 20 μg/ml (Fig. 4). This indicates that TFC treatment in MGC-803 cells can down-regulate survivin mRNA expression.

## 4. Discussion

Chalcones are precursor compounds for flavonoid synthesis in plants, and many reports have documented their biologically active properties (Dimmock et al., 1999; Go et al., 2005). Epidemiologic studies prove that intake of flavonoid and chalcones with

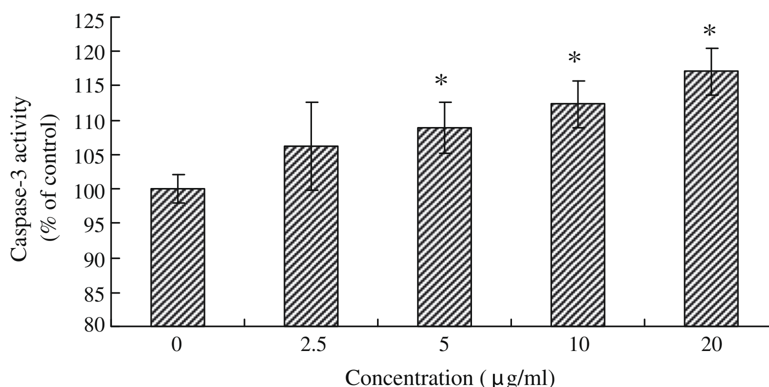


**Fig. 3.** TFC-induced caspase-3 activation in MGC-803 cells. Cells were incubated with TFC for 48 h. Caspase-3 activity was measured as described in Section 2. Values were expressed as a percentage of control. Untreated cells were designated as 100%. Data are means ± S.D. *$P < 0.05$ compared to control (percentage of control).

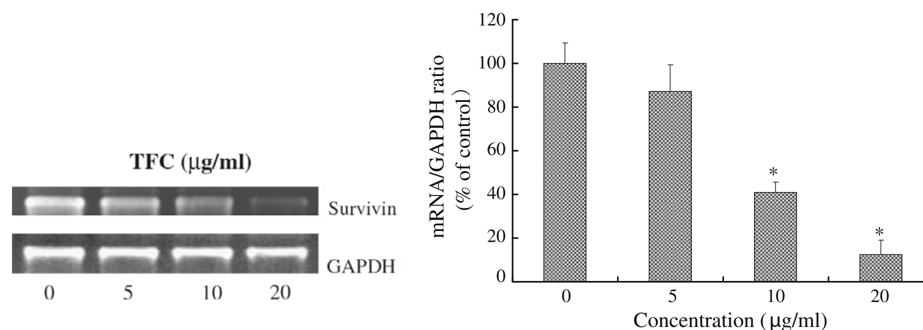


**Fig. 4.** Survivin mRNA expression in MGC-803 cells treated with TFC. Amplified DNA was electrophoresed through 1.5% agarose gels and visualized by staining with ethidium bromide. Results are representative of at least three independent experiments showing similar results. Graph represents band density ratio of each mRNA to GAPDH. Data are means ± S.D. $^{*}P < 0.05$.

fruits and vegetables could lower the prevalence of cancer in humans (Stoner and Mukhtar, 1995; Yang et al., 2001). In our previous work, a preliminary study on the antitumor activity of TFC was conducted (Lou et al., 2009). In this report, we determine that (i) TFC induces the apoptosis of MGC-803 cells by Hoechst 33258 staining (Fig. 2) and (ii) TFC (0–10 μg/ml) results in a dose-dependent G2/M phase cell cycle arrest of MGC-803 cells, though effects diminish at high dose (20 μg/ml) (Table 1).

To elucidate the molecular mechanism of the apoptotic role of TFC in MGC-803 cells, survivin expression was examined by RT-PCR. TFC appears to induce apoptosis in MGC-803 cells and to regulate survivin expression. TFC induces the decrease of survivin mRNA (Fig. 4). To the authors' best knowledge, survivin is expressed in a cell-regulated manner in the G2/M phase of the cell cycle. The interaction of survivin with the mitotic spindle is essential for anti-apoptotic function (Li et al., 1998). Overexpression of survivin has oncogenic potential because it may overcome the G2/M checkpoint to enforce progression of cells through mitosis. Severe inhibition of survivin by TFC prevents cell cycle progression through the M phase, resulting in apoptosis. Consistent with this notion, TFC treatment blocked the cell cycle in the G2/M phase. The diminished effects at high dose (20 μg/ml) suggest that G2/M phase arrest is followed by apoptotic cell death, which results in the elimination of cells in the G2/M phase, thereby lowering the cell number. The down-regulation of survivin mRNA is therefore a very important mechanism, and the decrease of survivin mRNA participates mostly in TFC-induced apoptosis of MGC-803 cells. Furthermore, the increase in S-phase cell population when using 5 μg/ml TFC (44.72% versus 30.51% in control) demonstrates that TFC can induce S-phase cell cycle arrest at this concentration. TFC treatment at the concentration of 5 μg/ml possibly blocked the cell cycle in both the S and G2/M phases. However, diminished effects of the S-phase cell cycle arrest at high doses (10–20 μg/ml) on MGC-803 cells need to be explored further.

Cysteine aspartases (caspases), a protease family, are required for apoptosis induced by various stimuli (Krepela, 2001). Among mammalian caspases, caspase-3 is considered the main effector of caspases and has been identified as being activated in response to cytotoxic drugs (Krepela, 2001). Caspase-3 activation is an important step in the execution phase of apoptosis and its inhibition blocks cell apoptosis (Budihardjo et al., 1999). In the current work, caspase-3 activity is increased in a dose-dependent manner when MGC-803 cells are exposed to different TFC concentrations (Fig. 3). To the authors' knowledge, survivin can inhibit the activation of downstream effectors of caspase-3 (Shin et al., 2001). The increasing caspase-3 activity may be due to the down-regulation of survivin mRNA. However, whether or not survivin directly binds caspase-3 in the apoptosis of MGC-803 cells is not shown in this report. Furthermore, the alteration in caspase-3 activity induced by TFC (Fig. 3) is marginal although significant, possibly because the time frame selected is too long to have the maximum effect.

In conclusion, results demonstrate that TFC treatment in MGC-803 cells can induce apoptosis and down-regulate the expression of the anti-apoptotic gene survivin. TFC therefore induces the apoptosis of MGC-803 cells via down-regulation of survivin mRNA expression. This study may also provide a basis for developing antitumor drugs with increased potency for chemoprevention and chemotherapy for gastric cancer. However, more studies are required to determine the exact mechanisms of action and to explore genetic and signal transduction pathways. A study including protein levels is ongoing in the authors' laboratory to better understand the biological mechanisms underlying the antiproliferative effect of TFC.

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