Antitumor and apoptogenic effects of ferulic acid on cervical carcinoma cells

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Abstract
Cancer is a disease characterized by uncontrolled cell proliferation and metastasis that has significant health and social impact. Currently, available anticancer therapies are not sufficiently effective and development of novel therapeutic agents and treatment strategies is urgently needed. The present study aims to investigate the in vitro anticancer activity and the underlying mechanisms of ferulic acid (FA) against human HeLa cells. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), a phenolic compound, which is abundant in vegetables and fruits, has been reported to exert numerous pharmacologic activities. The presented data showed that FA inhibited the cell proliferation in a dose- and time-dependent manner. Moreover, FA induced cytopathic alterations in HeLa cells and cell death were characterized by morphology and chromatin condensation changes, of a typical apoptosis.

These findings suggested that FA could effectively act as anticancer agent causing death of HeLa tumor cells by induction of apoptosis and provide the possibility for future development of chemotherapeutics for treatment of cervical carcinoma.

Keywords: Ferulic acid, cervical carcinoma, antitumor activity, apoptosis.

Introduction
Dysregulation in cell proliferation and apoptosis is the hallmark of cancer development [9, 29]. Apoptosis is a process by which cells undergo programmed cell death under certain physiological or pathological conditions [6]. Cell signaling pathways regulating proliferation, differentiation and apoptosis have a profound effect on the progression of cancer and therefore are targeted by many antitumor therapeutics [7, 11, 19, 28]. The majority of anticancer drugs and chemopreventive agents restrain the promotion and progression of tumors through induction of apoptosis [21, 34, 39]. Recently, researchers have focused on screening of natural compounds that are able to induce apoptosis in tumor cells and could be used as novel anticancer agents with less unwanted side effects.

Ferulic acid is a phytoconstituent that exhibits diverse pharmacological and biochemical effects including antioxidant, anti-inflammatory, hepatoprotective, antidiabetic, immune-enhancing activity, anticancer and pro-apoptotic properties and has applications in food preservation.[3,15,17,18,20,27,32,33,41,42] A number of *in vitro* studies indicated that ferulic acid inhibits proliferation, suppresses migratory ability and induces apoptosis in wide variety of tumor cells including mammary carcinoma[32,41], colon carcinoma[14], pancreatic carcinoma[35], lung carcinoma[22], urinary bladder carcinoma[25] and osteosarcoma[38,40]. In animal experiments, ferulic acid was found to exert protective effect on chemically-induced skin and oral carcinogenesis[1,26] and to suppress the tumor growth in pancreatic and mammary xenograft models[35,41].

The aim of this study is to assess the *in vitro* anticancer effects of ferulic acid against human cervical carcinoma cells HeLa and to understand more about the underlying mechanisms of the antineoplastic activity.

Material and Methods
Ferulic acid: Ferulic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA), FA ($C_{10}H_{10}O_4$, MW 194.18, purity 99%). The compound was dissolved in DMSO and further diluted with sterile distilled water to obtain stock solution with a concentration of 40 mM. Working solutions of FA (8 mM; 4 mM; 2 mM; 1 mM and 0.5 mM) were prepared from the stock solution by dilution with complete culture medium. The DMSO concentration of the solutions used in the experiments did not exceed 0.5%.

Cell culture: A HeLa cell line was cultured at 37 °C in a humidified atmosphere with 5% CO_2 and maintained in DMEM medium supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco-BRL).

Cell viability assay: To assess cell growth inhibition, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used [24]. Briefly, cells at a cell density of 10^4 cells in 100μL medium /well were seeded in a 96-well plate and incubated overnight. Various concentrations of ferulic acid were added to the cells and incubated for 24 h, 48 h and 72 h. Control HeLa cells were incubated with culture medium only. After exposure of the cells to different ferulic acid concentrations, the medium was removed and the cells were incubated with MTT (Sigma Chemical Co., St Louis, MO) (0.5 mg/ml in PBS) for 4 h.

A lysing buffer (DMSO:Ethanol v:v 1:1) was used to dissolve the formazan crystals. Optical density was

measured at 540 nm using an ELISA reader (TECAN, Sunrise TM, Groedig/Salzburg, Austria). A reference wavelength used in this study was 620 nm. The production of formazan dye was proportional to the number of viable cells.

Light microscopy: In order to investigate the effects on cell morphology, HeLa cells treated with ferulic acid in concentrations ranging from 0.5mM to 8mM were daily examined under inverted microscope (Olympus) and photographs were taken at the 72th hour of treatment.

Fluorescent microscopy: The ability of ferulic acid to induce apoptosis in the HeLa tumor cells was assessed after staining of control and treated cells with acdidine orange and etidium bromide (AO/EtBr) and 4',6-diamidino-2-phenylindole (DAPI).

Double staining with acdidine orange and etidium bromide: Double intravital staining with AO/EtBr was used to assess the apoptosis-inducing activity of ferulic acid according to Wahab et al[36]. Briefly, HeLa cells were seeded at 2x10^5 cells/ml on glass cover slips placed in the bottom of 24-well plate, incubated for 24 h to obtain a monolayer and after that were treated for 24 h with ferulic acid with 2mM concentration (= IC_{50} established by an MTT test). At the end of the incubation, the cover slips were washed with PBS and stained using equal volumes (1:1 v/v) of AO and EtBr solutions (10 µg/mL). The morphology of the treated cells was compared to that of control HeLa cells under a fluorescence microscope (Leica DM 500B, Wetzlar, Germany).

DAPI staining: For DAPI (4',6-diamidino-2-phenylindole) staining, HeLa cells at a concentration of 2x10^5 cells/1 ml were seeded on glass cover slips and cultivated overnight in a CO_2 incubator to obtain a monolayer. The cells were then treated with FA in concentration approximating the IC_{50} value for 24 hr. Next, the slides were washed with phosphate-buffered saline (PBS), fixed with methanol for 5 min and stained with DAPI 1µg/mL methanol for 30 min at 37 ºC in the dark. Ultimately, the cells were washed with methanol and mounted on microscope slides with glycerol. The stained cells were visualized under a fluorescence microscope (Leica DM 500B, Wetzlar, Germany).

Statistical analysis: The data from three replicated experiments are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using One-way ANOVA followed by a Bonferroni's post hoc test (GraphPad Prism). The $p < 0.05$ was accepted as the lowest level for statistical significance. Nonlinear regression (curve fit) analysis (GraphPad Prism) was applied to determine the concentrations inducing 50% inhibition of the cell growth (IC_{50} values).

Results and Discussion

Measurement of cell survival of HeLa human cancer cells after 24 h, 48 h and 72 h exposure to ferulic acid was performed by an MTT assay, a conventional tetrazolium-based colorimetric cell proliferation assay. Ferulic acid inhibited the proliferation of the HeLa cancer cell line in a concentration- and time-dependent manner. A pronounced inhibition of the cell proliferation was observed in the cells treated with 2mM, 4mM and 8mM FA, whereas the lower concentrations of FA did not induce a significant reduction of the cell viability compared to the control (Fig. 1). The mean IC_{50} values determined after 24, 48 and 72 hours of treatment were 2.597 mM, 2.127 mM and 1.793 mM respectively.

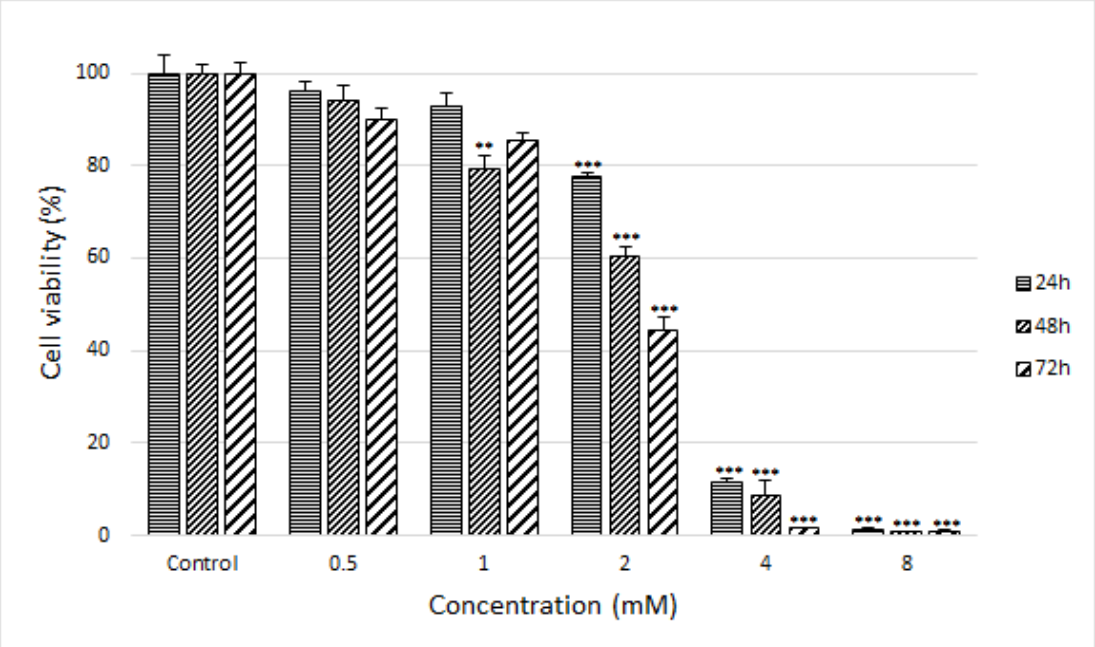

Figure 1: Antiproliferative effect of ferulic acid on HeLa tumor cells after 24, 48 and 72 hours of treatment.

Morphological changes in HeLa cells treated with different concentrations of FA were examined by light microscopy. FA-treated HeLa cells exhibited significant change in morphology and cytopathic alterations compared to the control. The observed alterations gradually aggravated with the increase of the concentration and the prolongation of the exposure time. The most significant changes were observed in cancer cells incubated with FA for 72h in concentrations higher than 2 mM – some of the cells were shrunk, rounded up and detached from the monolayer. FA in concentrations of 4mM/mL and 8mM/mL completely inhibited the proliferative activity of the tumor cells and hampered the formation of a cell monolayer (Fig. 2).

We also investigated whether the inhibition of proliferation by FA was achieved by apoptosis in the HeLa cells. Apoptotic cell death was assessed by acridine orange and ethidium bromide double staining (AO/EtBr).

AO and EtBr stain DNA allows visualization and differentiation of dead and viable cells. Cells with intact membranes are stained green due to the passage of AO, whereas EtBr stains only the cells with damaged membranes and the intercalation of both fluorescent dyes in DNA gives orange fluorescence.

No cell death was observed in the control HeLa cells (Fig. 3a). Considerable amount of damaged HeLa cells was noticed when exposed to FA (Fig. 3b). The cells cultured in the presence of FA displayed morphological signs of apoptosis such as shrinkage, membrane blabbing, chromatin condensation and margination, damaged wrinkled cells with orange fluorescence. These findings indicate that the induction of apoptosis is one of the major mechanisms through which FA exerts its action to destroy tumor cells.

The apoptosis-inducing potential of FA was further analyzed by DAPI staining. The results indicated that the number of apoptotic cells were higher in FA-treated cells than in untreated controls. The morphological changes that occurred in HeLa cells as a result of FA treatment are presented in fig. 4. Treatment with FA resulted in marked nuclear fragmentation and chromatin condensation, which is clear indication of apoptosis.

In the present study, a dose-dependent growth inhibition observed in the FA-treated cells indicates that FA is a potential cytotoxic agent against human HeLa cancer cells. The highest cytotoxic activity was observed in cell cultures treated with FA in concentrations higher than 1mM. The apoptosis-inducing potential of FA was determined by the morphological observation under an inverted microscope and after the AO/EtBr staining. Apoptosis is characterized by morphological features such as cell shrinkage, membrane blabbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies [31, 36].

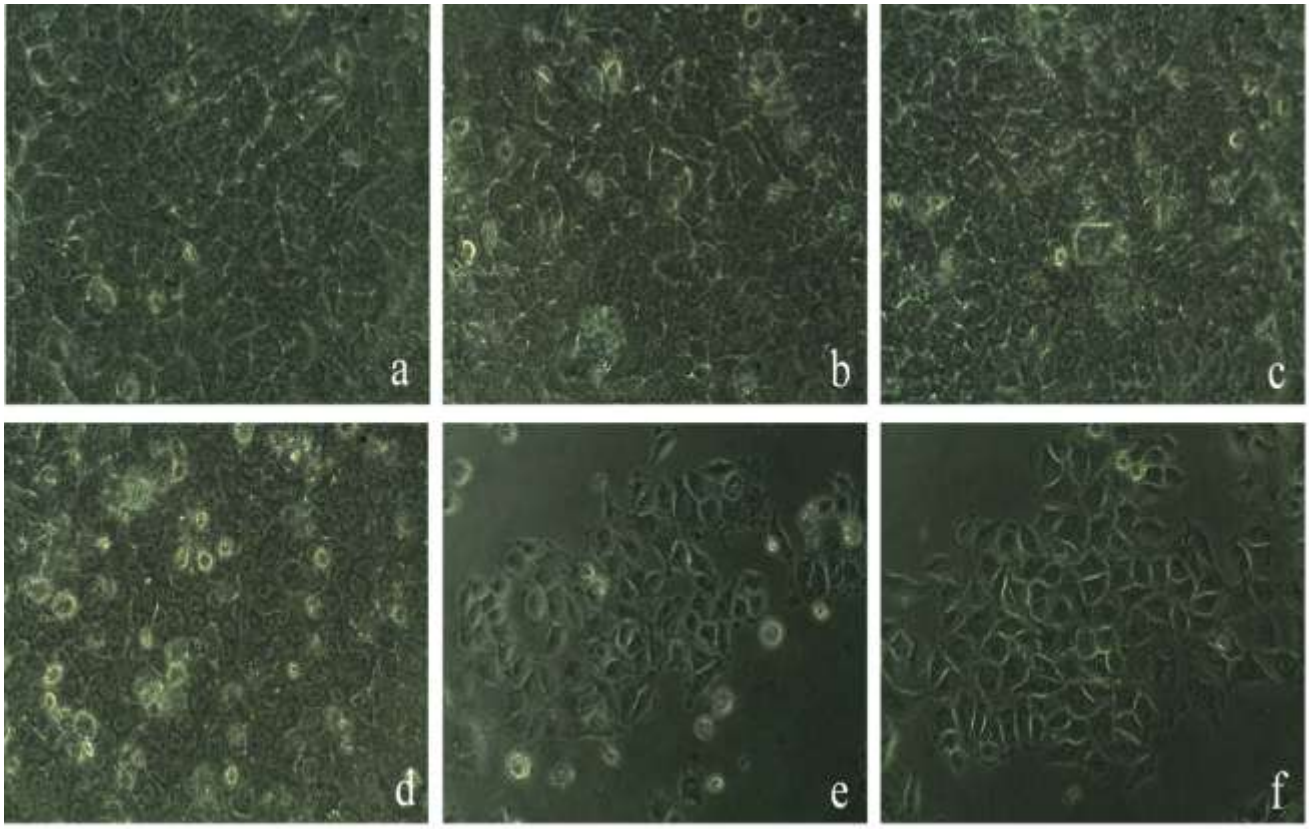

Figure 2: Morphological changes in HeLa tumor cells exposed to ferulic acid for 72h; a-control HeLa cells; b-, c-, d-, e-, f- HeLa cells treated with 0.5 mM, 1 mM, 2 mM, 4 mM and 8 mM FA. Light microscopy; Phase contrast; Objective 40X

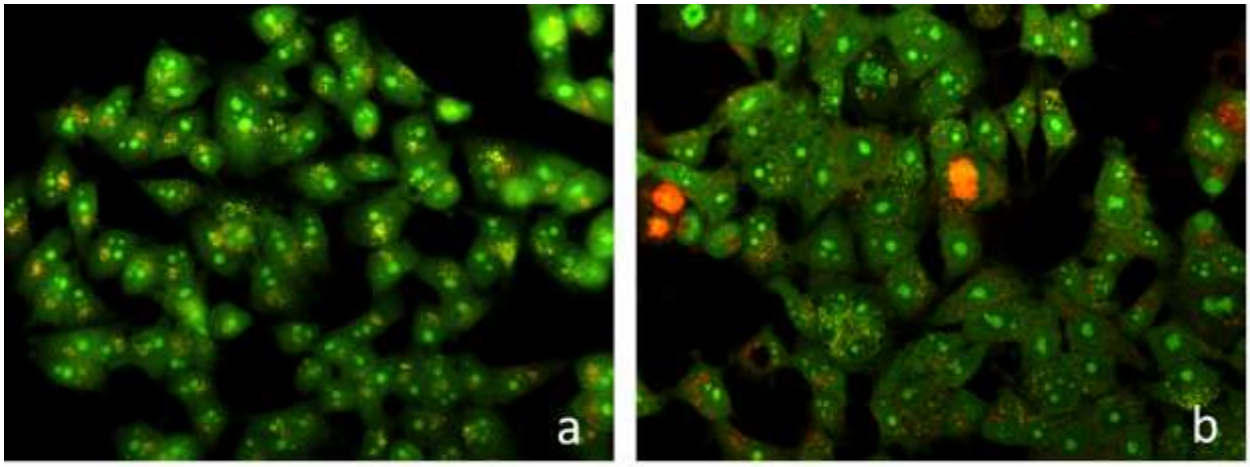

Figure 3: Fluorescent microscopy of HeLa tumor cells exposed to 2mM ferulic acid for 24 hours; a-control culture; b-culture treated with 2mM FA. Double staining with acridine orange and etidium bromide. Objective 40X.

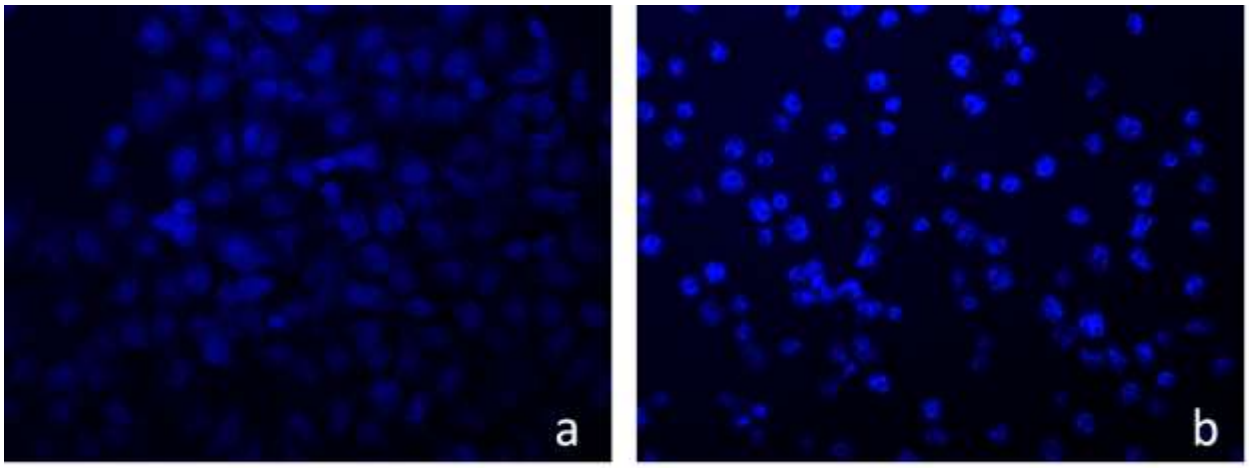

Figure 4: Fluorescence image of HeLa cells stained with 4′,6-diamidino-2-phenylindole (DAPI) after 24 h incubation with the 2mM FA. A- untreated cells, B- cells treated with 2mM FA. Objective 40X.

Several reports have shown that FA induced apoptosis in human tumor cells of various tissue origin[5,13-15,25,32,38,40]. Experiments with the human urinary bladder cancer cell line T24 revealed that FA induced different cytotoxicity and apoptotic rates in 2D and 3D culture systems[25]. Zhang et al[39,40] demonstrated the antitumor activity of ferulic acid *in vitro* and *in vivo* and confirmed that ferulic acid could suppress breast cancer migration and metastasis by inhibiting the epithelial to mesenchymal transition process. FA also inhibits the *in vitro* growth of colon cancer cells[14,23] as well as *in vivo* colon cancer development in rats[12,16].

Studies with laboratory animals have also shown suppressing activity of FA on oral and skin carcinogenesis[1-3,26]. The anti-carcinogenic activity of ferulic acid has been attributed to its antioxidant potential and capability of scavenging reactive oxygen species as well as stimulation of phase II detoxification enzymes[8,18,33].

Fluorescent microscopy of Hela cells stained with DAPI also indicated that FA possesses high apoptotic potential. DAPI staining is a reliable apoptotic assay that allows direct observation of the apoptotic changes in the cell nucleus[30]. In our current study, the chromatin condensation and nuclear fragmentation were observed in HeLa cells by DAPI staining after 24 h of treatment with the FA. These findings indicate that the cell death, which occurred, was not due to necrosis, but due to apoptosis. In addition, the results of the present study clearly correlate with earlier *in vitro* studies, which assayed the apoptotic potential of the FA in Hep-2 cell line and found similar nuclear alterations[27].

The previous studies conducted in different cancer cell lines suggest that FA decreases the cell proliferation and induces early apoptotic changes such as chromatin condensation, nuclear fragmentation, flipping of phosphatidylserine of the plasma membrane and decreased mitochondrial membrane potential[35,40].

Hou et al[10] reported that ferulic acid inhibited the proliferation of ECV 304 cells through increasing the production of nitric oxide (NO), which subsequently downregulated the extracellular signal-regulated kinase (ERK1/2) pathway[10]. Several reports in different tumor cell

lines indicate that FA significantly modulate the expression of apoptosis related proteins such as p53, Bcl-2, Bax, Caspase 3 and Caspase 9[4,25,27]. Our results are in line with the previously published data indicating a significant proapoptotic activity of FA in various tumor cell lines.

Conclusion

In conclusion, this study demonstrates the antiproliferative and apoptotic activity of FA in human HeLa cancer cells. The results confirmed that the directly suppressed proliferation of HeLa cells was associated to the induction of apoptosis. Our study confirmed that FA may be a novel candidate for cancer treatment. Further studies are needed to determine the *in vivo* antitumor effect of FA in experimental tumor models.

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