Fangchinoline Inhibits Breast Adenocarcinoma Proliferation by Inducing Apoptosis

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Radix Stephaniae tetrandrae, which contains tetrandrine (Tet) and fangchinoline, is traditionally used as an analgesic, antirheumatic, and antihypertensive drug in China. In this study, we investigated its effect on breast cancer cell proliferation and its potential mechanism of action in vitro. Treatment of cells with fangchinoline significantly inhibited MDA-MB-231 cell proliferation in a concentration- and time-dependent manner. To define the mechanism underlying the antiproliferative effects of fangchinoline, we studied its effects on critical molecular events known to regulate the apoptotic machinery. Specifically, we addressed the potential of fangchinoline to induce apoptosis of breast cancer cells. Fangchinoline induced internucleosomal DNA fragmentation, chromatin condensation, activation of caspases-3, -8, -9, and cleavage of poly(ADP ribose) polymerase, as well as enhanced mitochondrial cytochrome c release. Furthermore, fangchinoline increased the expression of the proapoptotic protein B cell lymphoma-2 associated X (Bax) and decreased the expression of the antiapoptotic protein B cell lymphoma-2 (Bcl-2). In addition, the proliferation-inhibitory effect of fangchinoline was associated with decreased levels of phosphorylated Akt. Our results indicate that fangchinoline can inhibit breast cancer cell proliferation by inducing apoptosis via the mitochondrial apoptotic pathway and decreasing phosphorylated Akt. Thus fangchinoline may be a novel agent that can potentially be developed clinically to target human malignancies.

Key words  fangchinoline; apoptosis; breast cancer

Apoptosis, or programmed cell death, is a normal physiological process of cell deletion in embryonic development, as well as in the maintenance of tissue and organ homeostasis. It is characterized by distinct morphological changes, including plasma membrane blebbing, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation. Inappropriate induction of apoptosis has been associated with organ injury, whereas failure to undergo apoptosis may cause cell overgrowth and malignancy. Various compounds can induce apoptosis due to their proapoptotic activity, and some of those compounds are now being considered as cancer chemopreventive and therapeutic agents.

Although many cellular proteins are involved in apoptosis, the B cell lymphoma-2 (Bcl-2) proteins are major regulators of apoptotic signaling. One of their roles is to control mitochondrial membrane integrity, thereby making them key regulators of caspase activation. Caspases are essential for the execution of cell death by apoptotic stimuli. The balance of antideath Bcl-2 proteins (e.g., Bcl-2, Bcl-xL, and Mcl-1) versus prodeath Bcl-2 proteins (e.g., Bcl-2 associated X (Bax), Bak) constitutes the life-death switch, and the prodeath BH3-only Bcl-2 proteins function by flipping this switch. The Akt pathway has been an important molecular target for cancer prevention and treatment. Activation of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway confers chemotherapeutic resistance in numerous tumor types.

Radix Stephaniae tetrandrae, the dried roots of Stephania tetrarindre S. Moore (Menispermaceae), are officially and traditionally used as an analgesic, antirheumatic, and antihypertensive drug in China. The main chemical constituents of radix Stephania tetrarindrae are tetrandrine and fangchinoline. In vitro assays have shown that fangchinoline inhibits histamine release and lowers blood pressure by functioning as a nonspecific calcium channel antagonist. Antinflammatory effects have also been observed in the mouse ear edema model. In addition, fangchinoline inhibits rat aortic vascular smooth muscle cell proliferation, cell cycle progression, and antitumor activity in human prostate carcinoma cancer cells and induced autophagic cell death but not apoptotic cell death in the HepG2 line. Fangchinoline was reported to be an antioxidant. However, there is little information in the literature on the cytotoxic effects of fangchinoline on human breast cancer cells. In the present study, we therefore investigated the antitumor effects of fangchinoline in breast cancer cells (Fig. 1) and its mechanism of action.

Experimental

Materials  Fangchinoline was purchased from the National Institutes for Food and Drug Control (Beijing, China) and was stable when stored at 4 °C. It was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and then stored at 4 °C. The final DMSO concentration did not exceed 0.1% (v/v), which had no influence on cell growth, in any experiment. Control cells were always treated with the same amount of DMSO (0.1%, v/v) as used in the corresponding experiments. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, and propidium iodide (PI) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Primary antibodies for caspase-3, caspase-8, caspase-9, poly(ADP ribose) polymerase (PARP), and Akt/p-Akt were purchased from Cell Signaling Technology (Beverly,

Fig. 1. Structure of Fangchinoline
MA, U.S.A.); antibodies for Bel-2, Bax, and cytochrome c were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture
Human breast cancer, MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). They were routinely maintained in Leibovitz’s L-15 medium (Gibco, Gaithersburg MD, U.S.A.), pH 7.3, supplemented with 100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 10% fetal calf serum (FCS) and grown at 37 °C in a humidified atmosphere of 5% CO2.

Cell Viability Assay
The cell viability of cancer cells treated with fangchinoline was determined using the MTT cell viability assay. Cells (4—5×10⁴) were seeded in 96-well plates and cultured for 24 h, followed by fangchinoline treatment for 48 h. A volume of 20 μl of MTT 5 mg/ml was added to each well and incubated for another 4 h at 37 °C. The medium was then aspirated carefully without disturbing the blue formazan crystals. DMSO was added (150 μl/well) for 15—20 min. Cells were incubated on an automated shaker. Light absorption (optical density (OD)) was measured at 490 nm on an enzyme-linked immunosorbent assay (ELISA) reader. The results were expressed as a percentage of the absorbance present in treated cells compared with control cells.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis
Cells were treated with fangchinoline or vehicle for 16 h. The cells were then harvested by trypsinization and washed once with phosphate buffered saline (PBS). Fragmented DNA was isolated using a DNA extraction kit (Beyotime Biotechnology, Hangzhou, China) according to the manufacturer’s instructions. Eluates containing the DNA were electrophoresed on 1% agarose gels containing 0.1% ethidium bromide. DNA band patterns were visualized under UV illumination.

Assessment of Cell Morphology
MDA-MB-231 cells were seeded at 5×10³ cells/well into 6-well flat-bottomed tissue culture plates and allowed to adhere overnight. DMSO vehicle or fangchinoline 20 μm was added to the cells, followed by incubation for 16 h. Cells were then washed with PBS, and stained with 10 mg/ml of Hoechst 33342 trihydrochloride dye in PBS for 20 min at 4 °C. The cells were then gently washed with PBS. The morphology of the nuclei was examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of Apoptosis by Flow Cytometry
Cells were seeded in 6-well plates at a density of 5×10³/well and cultured for 24 h, followed by fangchinoline and DMSO (control) treatment for 24 h. Following treatment, both adherent and nonadherent cells were harvested by trypsin digestion, washed twice with PBS, and resuspended in 0.5 ml of N-2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing NaCl 140 m M and CaCl₂ 2.5 m M. The cell suspension was mixed with 5 μl of annexin V-fluorescin isothiocyanate (FITC) (Molecular Probes, Eugene, OR, U.S.A.) and 10 μl of 10 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO, U.S.A.), incubated for 10 min in the dark at room temperature and then immediately analyzed with a flow cytometer (Becton Drive, Franklin Lakes, NJ, U.S.A.).

Western-Blot Analysis
After MDA-MB-231 cells were cultured in different fangchinoline concentrations for 24 h, they were washed three times with ice-cold PBS and lysed in radio immunoprecipitation assay (RIPA) buffer (Tris buffer 50 m M, ethylenediaminetetraacetic acid (EDTA) 5 m M, NaCl 150 m M, 1% NP 40, 0.5% deoxycholic acid, sodium orthovanadate 1 m M, aprotinin 81 μg/ml, leupeptin 170 μg/ml, and phenylmethylsulfonyl fluoride (PMSF) 100 μg/ml, pH 7.5). After mixing for 30 min at 4 °C, the mixtures were centrifuged (10000×g) for 10 min, and the supernatants were collected as whole-cell extracts. The extraction and isolation of nuclear and cytoplasmic protein were performed according to the instructions for the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). The supernatant was analyzed for protein concentration using the Bradford method (BioRad, Hercules, CA, U.S.A.). Equal amounts of cellular protein (30 μg/lane) were loaded onto a denaturing 8—12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a nitrocellulose membrane. The membrane was blocked overnight in nonfat powdered milk at 4 °C. After blocking, the membrane was probed with the appropriate primary antibody followed by a secondary horse-radish peroxidase (HRP)-conjugated antibody. Actin was used to normalize for protein loading. All experiments were performed at least twice with similar results.

Statistical Analysis
Data are expressed as mean±S.D. All data are expressed as a percentage compared with vehicle-treated control cells. Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA). t-test for independent or correlated samples. When appropriate, Student’s t-test for paired and correlated samples was applied. A p value of <0.05 (*) and <0.01 (**) or <0.001 (*** *) was considered to represent a statistically significant difference.

Results

Fangchinoline Inhibits Cancer Cell Proliferation
To determine the effects on cancer cell proliferation, MDA-MB-231 cells were treated with fangchinoline (6.25—100 μM). After 24 and 48 h, cell proliferation was determined in the MTT assay. Fangchinoline significantly decreased the percentage of viable cells as compared with control cells, and those effects were dose and time dependent (Fig. 2), with IC₅₀ values of 25.32±1.2 μM and 14.48±1.5 μM, respectively.

Fangchinoline Causes Cancer Cell Apoptosis
MDA-MB-231 cells were stained using Hoechst 33342. Control cells showed homogenous staining (Fig. 3A), whereas cells treated with fangchinoline (20 μM) for 16 h showed chromatin condensation (Fig. 3B). These observations indicate that the cells treated with fangchinoline entered apoptosis. We next evaluated the apoptotic effects of fangchinoline on MDA-MB-231 cells by measuring cellular DNA fragmentation. When the cells were treated with vehicle for 16 h, DNA fragmentation was not detectable. However, DNA fragmentation was apparent in cells treated with fangchinoline 20 μM at the 16 h time point (Fig. 3C). Fangchinoline-induced cell apoptosis was also evaluated in the annexin V-FITC/PI dual staining assay. To perform this assay, MDA-MB-231 cells were treated with 0, 5, 10, and 20 μM of fangchinoline for 24 h, then stained with annexin V-FITC and PI and subjected to flow cytometry. Fangchinoline dramatically enhanced the apoptotic population (annexin V-positive cells). The percentage of early apoptotic cells was 4.4%, 5.4%, and 14.8%, respectively. These results suggest that fangchinoline inhibits the proliferation of MDA-MB-231 cells by inducing apoptosis in a concentration-dependent manner (Fig. 4).

Fangchinoline Regulates Molecular Mechanisms of Apoptosis in MDA-MB-231 Cells
After cellular incubation with fangchinoline 5, 10, and 20 μM for 24 h, using a cleavage-specific PARP antibody, marked generation of the PARP fragment was observed after treatment of MDA-MB-231 cells (Fig. 5). In parallel, there was a dose-dependent loss of procaspase-3, -8, and -9 and a concentration-depen-
A dent increase in cleaved caspase protein (Fig. 6). Fangchinoline also caused the release of cytochrome c from mitochondria (Fig. 7A). Our results show that fangchinoline can potentiate apoptosis in MDA-MB-231 cells. To obtain a deeper insight into the mechanism of fangchinoline-mediated apoptosis in MDA-MB-231 cells, we examined the protein levels of Bcl-2 family members including Bcl-2 and Bax. Western-blot analysis showed that the expression of Bcl-2 significantly decreased after treatment with fangchinoline. In contrast, the expression of Bax was significantly increased (Fig. 7B). The results showed that fangchinoline clearly reduced phosphorylated Akt in a concentration-dependent manner (Fig. 8), indicating that fangchinoline inhibits Akt activities.
The levels of total Akt were not visibly changed, although the phosphorylated form was inhibited much more significantly.

Discussion

Tetrandrine and fangchinoline, a bisbenzylisoquinoline alkaloid isolated from the roots of *S. tetrandra*, have been shown over the past 10 years to possess antitumor activity in animal models and cultured tumor cells such as HepG2 cells, A549 human lung carcinoma cells, Neuro 2a mouse neuroblastoma cells, human hepatoblastoma cells, human colon carcinoma cells, the rat RT-2 glioma cell line, and the ECV304 human umbilical vein endothelial cell line. In the present study, we investigated the mechanism behind the antiproliferative effects of fangchinoline in breast cancer cells. Our study demonstrated that cancer cells treated with fangchinoline led to marked chromatin condensation and nuclear fragmentation, a biochemical hallmark of apoptosis. In addition, fangchinoline induced the release of (mono- or oligo-) nucleosomes into the cytoplasm and caused DNA laddering as well as leakage of degraded DNA from apoptotic nuclei, well-recognized processes that typically occur during apoptosis. Annexin/PI staining analysis confirmed that apoptosis was promoted in cancer cells treated with fangchinoline.

Among the family of caspases, caspase-3 has been reported to be the most frequently activated caspase protease in apoptotic cells, indicating its crucial role in the cell death process. Caspase-3 activation causes the cleavage or degradation of several important substrates, including PARP. In this study, fangchinoline effectively activated the expression of caspase 3 and cleaved PARP. Typically, caspase initiators, such as caspase-8 or caspase-9, were activated after interaction with receptors or mitochondrial release of cytochrome c. In turn, these initiator caspases activate executioner caspases, which then cleave cellular substrates leading to diverse and irreversible biochemical and morphological changes. Caspase-3 is activated by both caspase-8 and caspase-9 and is assumed to be a major executioner caspase. The initiation of apoptosis is through two distinct pathways: one is the extrinsic pathway involving receptor signaling; and the other is the intrinsic pathway involving the mitochondrial cascades.

To determine the pathway of apoptosis induced by fangchinoline, we further analyzed caspase-8 and -9. Our results showed that caspase-9 and caspase-8 were activated by fangchinoline. The release of mitochondrial cytochrome c is a signaling event in the intrinsic apoptotic activation pathway. Our results showed that cancer cells treated with fangchinoline released cytochrome c from the mitochondria into the cytosol.

Bel-2 family members are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability required for the release of cytochrome c. Our results showed that fangchinoline downregulated Bel-2 and upregulated Bax in cancer cells. The release of cytochrome c from the mitochondria into the cytosol by fangchinoline is a result of the downregulation of Bel-2 and the upregulation of Bax. Taken together, we conclude that fangchinoline induces apoptosis of MDA-MB-231 cells via an intrinsic mechanism that is regulated via the inhibition of Bel-2 and a concomitant stimulation of Bax protein expression. Akt regulates the process of cell survival by phosphorylating different substrates that directly or indirectly regulate the apoptotic program, such as inhibition of the proapoptotic Bel-2 family members. The present study demonstrated that fangchinoline treatment causes a decrease in activating phosphorylation of Akt in MDA-MB-231 cells. Therefore our results provide evidence that fangchinoline can be a potential agent against breast cancer by downregulating Akt activity.

In conclusion, we demonstrated that fangchinoline possesses potential anticancer activity and induces apoptosis in MDA-MB-231 cells in vitro. On the basis of these promising results, further investigations and clinical trials must be performed to determine its possible benefits to patients.

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References


