Fangchinoline inhibits migration and causes apoptosis of human breast cancer MDA-MB-231 cells

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Abstract. In order to improve outcomes after breast cancer treatment, it is essential to understand the mechanisms of action of potential therapeutic agents. The effect of fangchinoline (FAN) on migration and apoptosis of human breast cancer MDA-MB-231 cells and its underlying mechanisms were investigated. MDA-MB-231 cells were treated with different concentrations of FAN, growth inhibition rates were measured by MTT assay and morphological changes of apoptotic cells were observed by Hoechst staining. The wound-healing assay was used to determine of the effect of FAN on the migration of MDA-MB-231 cells. ELISA was used to detect the expression of MMP-2 and -9 in MDA-MB-231 cells treated with different concentrations of FAN and western blot analysis was used to quantify expression of NF-κβ and Iκβ proteins in the same cells. Our results showed that FAN significantly inhibited the growth of MDA-MB-231 cells in concentration-dependent manner and it induced MDA-MB-231 cell apoptosis. With the high FAN concentrations and long exposure times, the levels of MMP-2 and -9 decreased and the expression of NF-κβ decreased, while the expression of Iκβ protein increased. Based on these results, the antitumor effects of FAN on breast cancer cells can be explained at least partially by inducing apoptosis and inhibiting the migration of MDA-MB-231 cells.

Introduction

Breast cancer is a common cancer in women. Its incidence has been increasing and the age of onset has been decreasing in previous years (1). Surgical tumor resection is frequently used to treat breast cancer, but recurrences are common and the trauma associated with the procedure is substantial. In addition, traditional radiotherapy can lead to adverse reactions. It is under this context that Chinese medicine and targeted gene therapies have become the focus of present studies aimed at improving the treatment against breast cancer. The main active ingredients of *Stephania tetrandra* include tetrandrine and fangchinoline (FAN) (2). Studies have confirmed that FAN can inhibit breast cancer cell proliferation; it does so by inhibiting the proliferation of breast cancer cells, inducing cell apoptosis mediated by mitochondrial pathways and reducing the level of phosphorylated AKT (3,4). However, other antitumor effects of FAN on breast cancer cells and their possible mechanisms have not been reported. The collagenases MMP-2 and -9 are usually involved in the metastasis of breast cancer by degrading the extracellular matrix (ECM). High expression levels of activated NF-κβ are also associated to metastatic breast cancer cells. This study aimed at investigating the possible effects of FAN on the migration of breast cancer cells and on their expression of MMP-2 and -9 and the activation of NF-κβ, so as to provide a basis for further clarification of the effects of FAN.

Materials and methods

Cell culture. Human breast cancer cell line MDA-MB-231 from the Cell Bank of the Chinese Academy of Sciences (Beijing, China) was cultured in L-15 medium supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin and containing 10% fetal bovine serum (all from Gibco Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), the incubator was set at 37˚C with 5% CO_2.

FAN (CAS: 33889-68-8, HPLC ≥98%, molecular formula: C37H40N2O6, molecular weight: 608.20 mg) was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). It was stored at 4˚C or dissolved in dimethyl sulfoxide (DMSO) to make a stock solution; the DMSO concentration used was lower than 0.1% to avoid deleterious effects on cell growth, the same final concentration of DMSO was also used for treatment in the control group cells.

Methyl thiazolyl tetrazolium (MTT) assay to detect cell proliferation. MDA-MB-231 cells were inoculated into 96-well plates with 4-5x10³ cells per well. After incubation for 24 h different concentrations of FAN (0.25, 12.5, 25, 50, and 100 µg/ml) were added to different wells. Next, 10 µl of

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a 5 mg/ml MTT solution (Sigma-Aldrich; Merck & Co., Inc., Whitehouse Station, NJ, USA) was added at 24 and 48 h after adding FAN. After incubation for another 4 h (at 37˚C), the supernatant was removed and 150 μl DMSO (Sigma-Aldrich; Merck & Co., Inc.) were added into each well, followed by incubation for 15 min. The OD value of each well was measured at 490 nm using a microplate reader (Puyun Biotechnology Co., Ltd., Jiangsu, China). The percentages of the OD values of the samples to a blank control were recorded.

Hoechst 33342 fluorescent staining to observe morphological changes of apoptotic cells. MDA-MB-231 cells were inoculated onto 6-well plates with 5×10^5 cells per well. After overnight incubation, DMSO for the blank control and 20 μg/ml FAN were added, followed by incubation for 16 h. After washing with phosphate-buffered saline (PBS), 10 mg/ml Hoechst 33342 staining solution was added and incubated at 4˚C for 20 min. After washing with PBS, the nuclear morphology was observed under a fluorescent inverted microscope (Shanghai Cai Kang Optical Instrument Factory, Shanghai, China).

Hoechst-PI double-staining. MDA-MB-231 cells were inoculated onto 6-well culture plates with 5×10^5 cells per well. DMSO for a blank control and 20 μg/ml FAN were added and incubated for 12, 24 or 48 h. After washing with PBS 3 times, 1 ml Hoechst 333428 staining solution was added and incubated at 20˚C for 15 min, after washing with PBS for 2 times, 50 mg/ml propidium iodide (PI) dye (Sigma-Aldrich; Merck & Co., Inc.) were added and incubated at 20˚C in the dark for 30 min. Flow cytometry was used to detect the fluorescence intensity, and to calculate cell apoptosis rate.

Cell migration assay. A marker pen was used to draw parallel lines on the reverse side of the 6-well culture plate so that each well was crossed through by at least 5 lines. The MDA-MB-231 cells were collected at logarithmic growth phase and inoculated with 2 ml of medium with 4×10^6 cells per well. After incubation in a sterile incubator for 48 h, a 200 μl tip was used to scratch the cells along the parallel lines. The cells were washed 3 times with PBS to remove all dislodged cells. Serum-free medium and 0, 5, 10 and 20 μg/ml FAN were added to different wells and photos were taken 0, 6, 12 and 24 h later. The ImageJ software (version X; Media Cybernetics, Silver Springs, MD, USA) was used to process the images.

The detection of MMP-2 and -9 content. MDA-MB-231 cells were seeded on 6-well plates with 5×10^5 cells per well. After overnight incubation, different concentrations of FAN (1, 50 and 100 μg/ml) were added to different wells and the plates were further incubated for 24 and 48 h. After incubation, the supernatant was collected. The supernatant was centrifuged and the levels of MMP-2 and -9 were measured by enzyme-linked immunosorbent assay (ELISA) according to the instructions on the kit (Dongge Biotechnology, Beijing, China). The OD values were measured at 450 nm using a microplate reader to calculate the concentration of MMP-2 and -9.

Western blot analysis. MDA-MB-231 cells were incubated with different concentrations of FAN (0, 5, 10 and 20 μg/ml)

Table I. Effect of FAN on the proliferation of MDA-MB-231 cells.

Groups	FAN concentrations (μg/ml)	Cell viability	
		24 h	48 h
Control	0	100.00±1.18	100.00±1.38
FAN	6.25	97.40±0.69	54.34±4.64[a]
	12.5	55.67±4.70[a]	40.60±6.42[a]
	25	26.57±5.06[a]	33.43±2.61[a]
	50	26.40±1.50[a]	23.83±1.45[a]
	100	24.57±3.19[a]	21.70±1.30[a]

[a]Compared with the control group, P<0.05; FAN, fangchinoline.

for 24 h. After that, the cells were washed thrice with pre-cooled PBS. Lysis RIPA buffer was the added and incubated for 30 min followed by centrifugation for 10 min at 4˚C to collect the supernatant, which contained the total soluble protein. The nuclear and cytoplasmic proteins were extracted according to the instructions on the kit (Beijing Huaxia Yuanyang Technology Co., Ltd., Beijing China). Protein concentrations were measured by the Bradford method; 30 μg of each protein sample were subjected to 8-12% polyacrylamide gel electrophoresis, followed by tranfer to a nitrocellulose membrane. The membrane was then blocked with 5% skim milk for 1 h followed by incubation with primary rabbit polyclonal NF-$\kappa\beta$ antibody (dilution, 1:500; cat. no. ab16502) and rabbit monoclonal I$\kappa\beta$ antibody (dilution, 1:500; cat. no. ab32518) overnight at 4˚C. After washing, secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721) was added and the signals were detected. At least two batches of similar results were obtained for each experiment and GAPDH was used as the endogenous control to normalize quantities. All antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis. Data were processed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) software. Measurement data are expressed as mean ± standard deviation and processed by t-test; count data are expressed as percentages and processed using χ^2 test. A P<0.05 was considered to indicate statistically significant difference.

Results

The cell proliferation assays showed that, compared with the control group, the percentages of viable cells in the groups treated with different concentration of FAN (6.25, 12.5, 25, 50 and 100 μg/ml) for different treatment periods (24 and 48 h) were significantly reduced in a time- and concentration-dependent manner (Table I). The IC$_{50}$ at 24 and 48 h was 14.26±1.54 μg/ml and 10.48±1.38 μg/ml, respectively.

The results of MDA-MB-231 cell staining with Hoechst 33342 showed that cells in the control group were homogeneously stained (Fig. 1A), while the cells treated
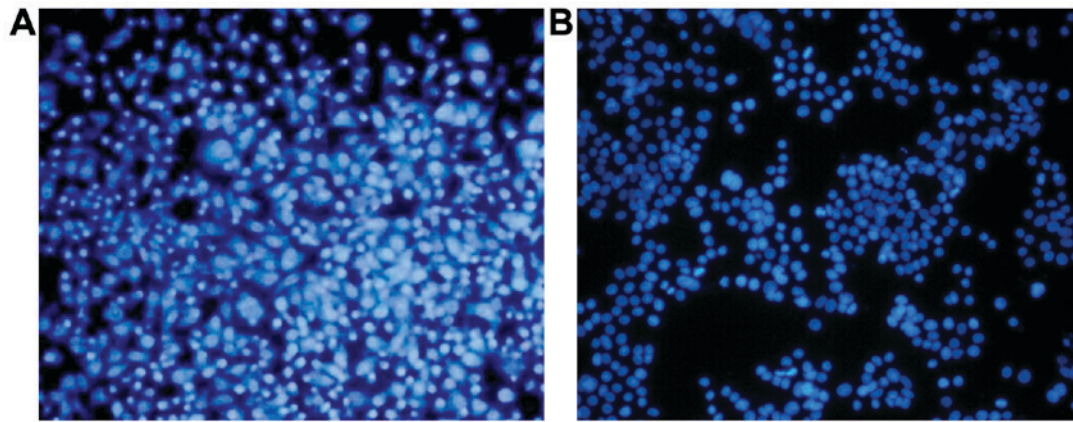
Figure 1. Hoechst staining showing morphology of MDA-MB-231 cells treated with FAN. (A) Control group showing homogeneous staining. (B) Cells with treatment of FAN (20 µg/ml) for 16 h showing nuclear shrinkage and fragmentation. FAN, fangchinoline.
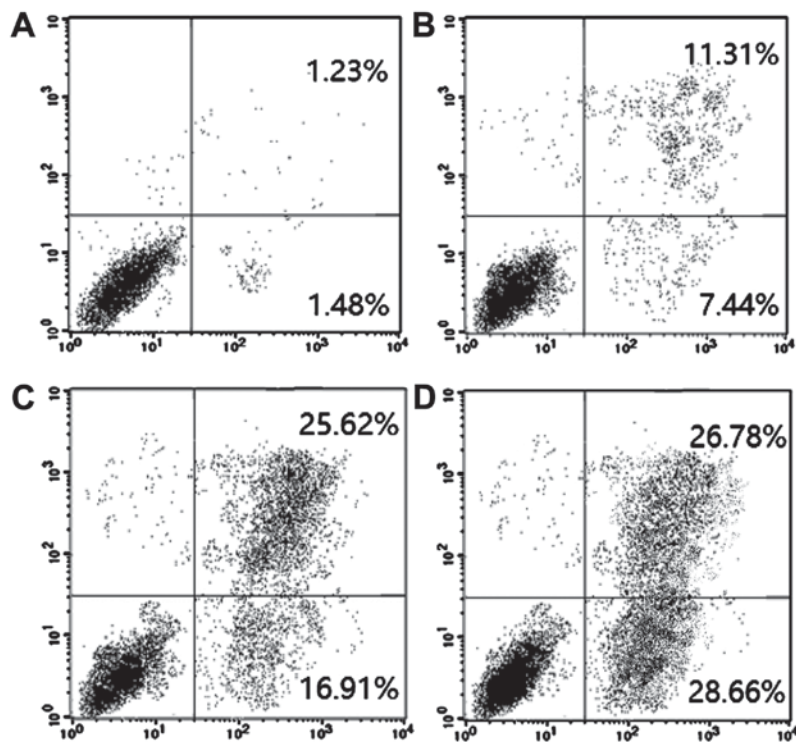

Figure 2. Apoptosis detected by flow cytometry on MDA-MB-231 cells treated with different concentrations of FAN. (A) Treatment with 0 µg/ml FAN, the apoptosis rate was 1.39%. (B) Treatment with 20 µg/ml FAN for 12 h, the apoptosis rate was 7.16%. (C) Treatment with 20 µg/ml FAN for 24 h, the apoptosis rate was 16.38%. (D) Treatment with 20 µg/ml FAN for 48 h, the apoptosis rate was 28.04%. FAN, fangchinoline.

with 20 µg/ml FAN for 16 h exhibited nuclear shrinkage and fragmentation (Fig. 1B), indicating that FAN treatment increased the apoptosis of MDA-MB-231 cells.

Furthermore, flow cytometry results showed that the apoptosis rate was increased with time. The apoptosis rates of MDA-MB-231 cells were 1.39, 7.16, 16.38 and 28.04% after 20 µg/ml FAN treatment for 0, 12, 24 and 48 h, respectively (Fig. 2).

Effects of FAN on cell migration by wound healing assay. The results of wound healing assays showed that the inhibition of FAN on cell migration was both dose- and time-dependent. The migration of the MDA-MB-231 cells treated with different concentrations of FAN (0, 5, 10 and 20 µg/ml) for 24 h was significantly inhibited. Increasing FAN concentrations led to progressively less migration (Fig. 3).

ELISA detection of MMP-2 and -9. The effect of FAN on the expression of MMP-9 and -2 was detected at 24 and 48 h after FAN treatment using ELISA kits. The results showed that the expression of MMP-9 and -2 decreased significantly with increasing FAN concentrations (Table II).

Western blot analysis to detect expression of NF-κβ and Iκβ in MDA-MB-231 cells. The western blot analysis showed that, after treatment with different concentrations of FAN for 24 h,

Table II. The effect of FAN on the expression of MMP-9 and -2 in MDA-MB-231 cells quantified by ELISA.

Groups	Concentration (μg/ml)	MMP-2 (pg/ml)		MMP-9 (pg/ml)	
		24 h	48 h	24 h	48 h
Control	0	318.76±12.58	317.89±12.73	1022.73±71.53	1023.16±72.53
FAN	1	297.48±9.19[a]	284.34±9.62[a]	967.40±70.39[a]	951.37±64.54[a]
	50	275.63±8.73[a]	262.43±7.49[a]	945.67±64.57[a]	938.6 ±66.72[a]
	100	266.57±7.16[a]	233.69±7.73[a]	926.57±65.86[a]	903.42±59.31[a]

[a]Compared with control group, P<0.05; FAN, fangchinoline; ELISA, enzyme-linked immunosorbent assay.
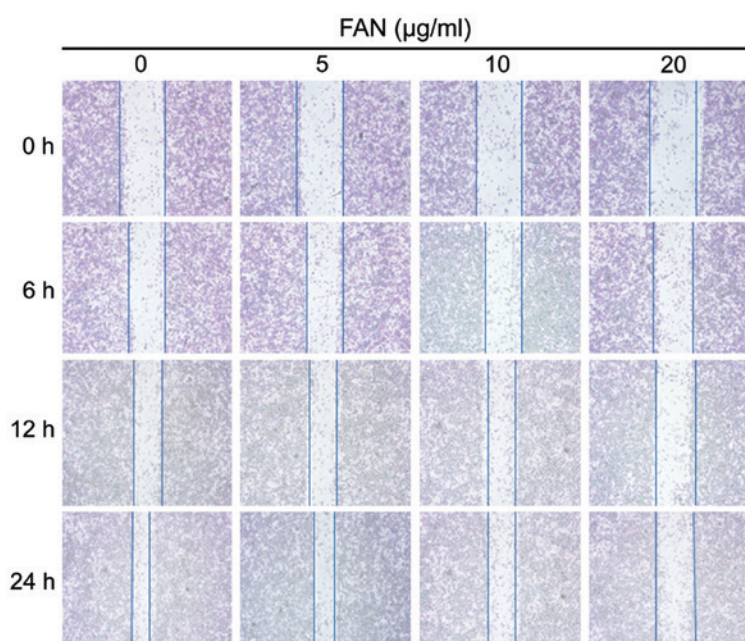

Figure 3. Wound healing assay showing effects of FAN on cell migration. Increasing FAN concentrations led to progressively less migration. FAN, fangchinoline.

the expression levels of NF-κβ protein were reduced and the expression levels of Iκβ were increased in a concentration-dependent manner (Fig. 4).

Discussion

A variety of contributing factors including quality of sleep, history of smoking and drinking, age at menarche, gravidity, age at menopause, waist and hip ratio and the history of breast disease can lead to the occurrence of breast cancer and breast cancer is associated with abnormal hormonal changes (1). The incidence of breast cancer is relatively high in two age groups: 45-50 and 60-65 years. The breast cancer in the first group is associated with reduced ovarian function and increased activity of the anterior pituitary, eventually leading the release of excessive estrogen from the adrenal cortex. While the occurrence of breast cancer in the latter group is associated with excessive androgen released by the adrenal cortex (1). Early diagnosis and treatment of breast cancer can significantly extend the survival of breast cancer patients and improve their quality of life.

FAN, which is a type of dibenzyl-isoquinoline alkaloid extracted from the roots of the *Stephania tetrandra*, is the main active ingredient of the plant (5). FAN has showed strong anticancer activities in a variety of tumor cell lines, including human HepG2, human lung cancer A549, murine neuroblastoma, human hepatoblastoma, human colon cancer cells and others (2). MDA-MB-231 cells are the most commonly used cells in breast cancer research. FAN can inhibit the proliferation and induce the apoptosis of MDA-MB-231 cells. FAN can also induce cell cycle arrest of breast cancer cells and affect the expression of cell cycle related proteins (6,7).

The results of this study show that the treatment of MDA-MB-231 cells with FAN led to nuclear shrinkage and fragmentation, which are the biochemical markers of apoptosis. The MTT assays showed that MDA-MB-231 cells treated with different FAN concentrations for up to 48 h showed a significantly reduced percentage of viable cells in a concentration- and time-dependent manner. The IC$_{50}$ at 24 and 48 h was 14.26±1.54 μg/ml and 10.48±1.38 μg/ml, respectively, which indicated that FAN did effectively inhibit the proliferation of breast cancer cells.

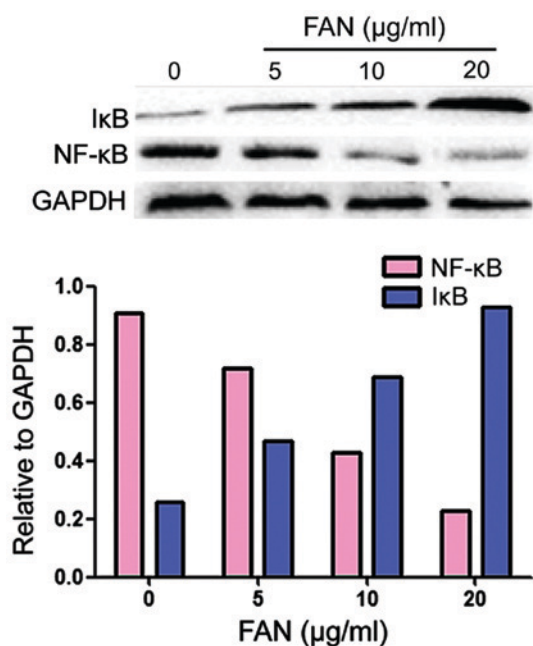Figure 4. Expression levels of NF-κβ and Iκβ protein in MDA-MB-231 cells after treatment with different concentrations of FAN (0, 5, 10 and 20 μg/ml) detected by western blot analysis. The protein level of NF-κβ was decreased and the protein level of Iκβ was increased in a concentration-dependent manner. FAN, fangchinoline.

FAN is known to inhibit the growth and induce the apoptosis of MDA-MB-231 cells, by activating the apoptosis-related protein caspase-3 (8,9). Our results showed that the apoptosis rates of MDA-MB-231 cells were 1.48, 7.44, 16.91 and 28.66%, respectively, after 20 μg/ml FAN treatment for 12, 24 and 48 h. A possible explanation is that FAN treatment can increase the activity of intracellular caspase-3 in MDA-MB-231 cells, thereby promoting MDA-MB-231 cell apoptosis.

Many studies have shown that degradation of matrix metalloproteinases (MMPs) play an important role in tumor invasion and metastasis. MMP-2 and -9, which are two members of MMPs family secreted by macrophages, neutrophils, capillary endothelial cells and tumor cells, are enzymes responsible for the degradation of the ECM. MMP-2 and -9 can be involved in the invasion and metastasis of colorectal, breast, gastric and other types of cancers by degrading ECM and have been specially studied in the invasion and metastasis of breast cancer (10,11). The expression of the MMP genes is mainly regulated by transcription factors such as NF-κβ and AP-1 through PI3K/AKT pathway (12). PI3K/AKT is a constitutive mutant gene pathway in most types of tumors. This pathway also regulates many extracellular processes, including tumorigenesis, cell proliferation, survival, glucose metabolism, gene stability, metastasis and angiogenesis (13). The results of this study show that different concentrations of FAN can significantly reduce the concentration of MMP-2 and -9 levels, thus potentially blocking the tumor cell metastasis.

As a universal transcription factor, NF-κβ is usually associated with viral infection and inflammation (14). The activated NF-κβ, which can promote the expression of angiogenesis-related factors such as VEGF, MMPs, plasminogen activator urokinase (u-PA) and ICAM-1, is closely related to the metastasis of tumor cells (15). Studies have shown that NF-κβ is overexpressed in breast cancer tissues (16). NF-κβ is regulated by Iκβ in the cytoplasm. After release, NF-κβ can enter the nucleus to promote tumor cell proliferation, neovascularization and metastasis (17). The activation of NF-κβ can cause the degradation of Iκβ through PI3K/Akt pathway. Some studies have shown that FAN can downregulate the PI3 K/AKT pathway to induce the apoptosis and inhibit the migration of breast cancer cells (18). The results of this study showed that the protein levels of NF-κβ were decreased and the protein levels of Iκβ were increased in a concentration-dependent manner after treatment with different concentrations of FAN for 24 h. It is therefore conceivable that FAN can inhibit the PI3K/AKT pathway, thereby increasing the concentration of Iκβ and that the increased in the Iκβ level can block the NLS of NF-κβ, so as to keep the NF-κβ in the cytoplasm in an inactive form, which would in turn inhibit the activity of NF-κβ (19). In addition, Iκβ can regulate the nuclear translocation of NF-κβ, thus affecting the expression of MMP-2 in multiple types of human cells (20).

In conclusion, based on our results we propose the following mechanisms explaining the effects of FAN on MDA-MB-231 cells: FAN inhibits the activation of AKT to increase the level of Iκβ. The increased concentration of Iκβ in the cytoplasm will then inhibit the activation of NF-κβ and then decrease the expression of MMP-2 and -9, so as to inhibit migration of the cells. Further studies are necessary to confirm the usefulness of FAN as an effective and safe anti-breast cancer drug.

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