RESEARCH ARTICLE

Fangchinoline Inhibits Cell Proliferation Via Akt/GSK-3beta/cyclin D1 Signaling and Induces Apoptosis in MDA-MB-231 Breast Cancer Cells

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Abstract

Fangchinoline (Fan) inhibits cell proliferation and induces apoptosis in several cancer cell lines. The effects of Fan on cell growth and proliferation in breast cancer cells remain to be elucidated. Here, we show that Fan inhibited cell proliferation in the MDA-MB-231 breast cancer cell line through suppression of the AKT/Gsk-3beta/cyclin D1 signaling pathway. Furthermore, Fan induced apoptosis by increasing the expression of Bax (relative to Bcl-2), active caspase 3 and cytochrome-c. Fan significantly inhibited cell proliferation of MDA-MB-231 cells in a concentration and time dependent manner as determined by MTT assay. Flow cytometry analysis demonstrated that Fan treatment of MDA-MB-231 cells resulted in cell cycle arrest at the G1 phase, which correlated with apparent downregulation of both mRNA and protein levels of both PCNA and cyclin D1. Further analysis demonstrated that Fan decreased the phosphorylation of AKT and GSK-3beta. In addition, Fan up-regulated active caspase3, cytochrome-c protein levels and the ratio of Bax/Bcl-2, accompanied by apoptosis. Taken together, these results suggest that Fan is a potential natural product for the treatment of breast cancer.

Keywords: Fangchinoline - proliferation - CyclinD1 - apoptosis

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Introduction

Despite the considerable progress in both the diagnosis and treatment of breast cancer, the disease remains the second leading cause of cancer-related deaths among women. Drug therapies directed at the estrogen receptor, the progesterone receptor, and human epidermal growth factor 2 receptor (HER2/neu) have resulted in improved clinical outcomes in some breast cancers. However, it is still a formidable challenge to treat those tumors that do not express the aforementioned molecular targets (Sorlie et al., 2001; Sørlie et al., 2003; Schneider et al., 2008). Fangchinoline (Fan) is a bis-benzylisoquinoline alkaloid, extracted from the Chinese medicinal herb Radix Stephania tetrandrae, dry roots of Stephaniae tetrandrine S. Moore (Menispermaceae). Fan shares structural features with tetrandrine, another functional compound extracted from Radix Stephania tetrandrae. Tetrandrine has been shown to be a potent calcium channel blocker and demonstrates anti-tumour activity in various cancer cells (King et al., 1988). Hsu et al reported that tetrandrine could stimulate opioid receptors which increased glucose utilization and/or reduced hepatic gluconeogenesis to lower plasma glucose levels in streptozotocin-diabetic rats (Hsu et al., 2004). In the last few years, great progress has been made assesssing the pharmacological role of Fan in the treatment of several diseases. For example, Fan has been shown to inhibit the release of glutamate from rat cerebral cortex nerve terminals and appears to possess neural protective properties (Lin et al., 2009). Moreover, Fan demonstrates anti-inflammatory effects (Choi et al., 2000) and anti-oxidant activity (Gulcin et al., 2010).

Recently, it was found that Fan inhibited both cell proliferation and cell cycle progression of rat aortic vascular smooth muscle cells by inhibiting the activation of ERK1/2 and expression of c-fos (Zhang et al., 2003). These data suggest that Fan could be a potential natural product for the treatment of breast cancer. In this study, we explored the anti-proliferative effects of Fan on MDA-MB-231 breast cancer cells, and found that Fan inhibited cell proliferation and induced cell apoptosis through inhibition of the Akt/GSK-3beta/cyclin D1 signaling pathway.

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Materials and Methods

Cells and cell culture

The MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC). Cells were seeded at a density of 1×10^4 cells/cm^2 and maintained in DMEM growth media supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C.

Cell proliferation assay

Cell proliferation was determined using the MTT assay (Promega). Cells were seeded at a concentration of 5000 cells/well in 24-well plates and incubated for 1, 2, 3, 4 and 5 days. At each time point, 100 µl media and 20 µl MTT was added to each well. Following incubation at 37°C for 4 hours, absorbance at 490 nm was detected using the spectrometer.

Flow cytometry

MDA-MB-231 cells (2×10^6) were plated in 100-mm plates with 15 ml of media, with or without Fan. After 2 days, the cells were re-suspended in PBS containing 1% Triton X, 0.1 mg/ml RNase A, and 0.05 mg/ml propidium iodide. The cells were subjected to FACS caliber flow cytometry, and the percentage of cells in each phase of the cell cycle was obtained using Modfit software.

Quantitative RT-PCR

Total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Reverse transcription of total RNA was carried out at 42 °C for 50 min and then at 70 °C for 15 min using the SuperScriptTM first strand synthesis system (Invitrogen). The resulting single-stranded cDNA was amplified by PCR using primers specific for proliferating cell nuclear antigen (PCNA) (forward primer, 5'-GAAGCCACCCACACCATCAC-3'; reverse primer, 5'-TTCTTCAAAAATCTGAC CATTCCAA-3'), CyclinD1 (forward primer, 5'-GAGGAGCAGCTCGCCA A-3'; reverse primer, 5'-CTGTCAAGGTCCGGCCAGC G-3'), and beta-actin (forward primer, 5'-TGGTCCT CTGGGCATCTCAGGC-3'; reverse primer, 5'-GGTGAA CCTGCTGTTGCCCTCA-3'). Amplification conditions included one cycle at 95°C for 3 min, followed by 34 cycles of 56°C for 1 min, and 72°C for 90 seconds, with a final elongation cycle at 72°C for 5 min. For semi-quantitative PCR analysis, PCR-amplified products were separated by 1.2% agarose gel electrophoresis and observed under ultraviolet illumination after staining with ethidium bromide. To estimate the degree of each transcript, relevant bands were quantified using the NIH Image J software and normalized against beta-actin. Three independent experiments were performed in duplicate, to estimate the relative amount of each gene product. The resulting data were expressed as mean ± SE.

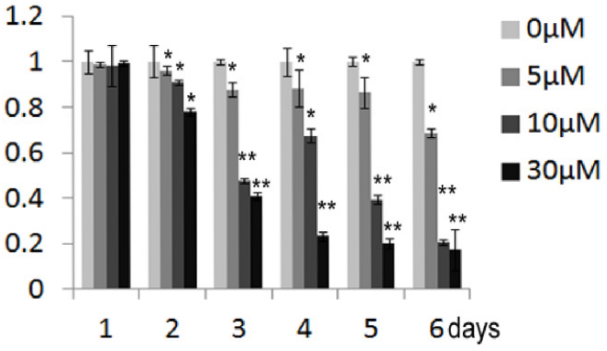

Figure 1. Fan Inhibited the Proliferation of MDA-MB-231 Cells. (A) MTT detection of the proliferation of MDA-MB-231 cells. MDA-MB-231 cells were seeded at 5000 cells/well in 96-well plates and treated with different concentrations of Fan (5 µM, 10 µM and 30 µM) for the indicated days. Cell proliferation was determined by the MTT assay, the y axis represent cell proliferation percent of control

Western blot analysis

Cells were lysed on ice with NP40 buffer (1% NP-40, 0.15 M NaCl, 50 mM Tris, pH 8.0) containing protease inhibitors (Sigma). Cell lysates were centrifuged at 12, 000 g for 10 min at 4°C, and the supernatants were stored at -80°C. Protein quantification was performed with the BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein from different groups were denatured in SDS sample buffer and separated on 8-10% SDS polyacrylamide gel, based on the protein molecular weight. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked with Tris-buffered saline (TBS; 137 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.1% Tween 20 and 5% dried milk powder. Mouse monoclonal antibody to Bax (Santa Cruz), rabbit polyclonal antibodies to Bcl-2, cytochrome-c, p21, AKT, GSK-3beta, p-AKT and p-GSK-3beta and beta-actin (Santa Cruz), and rabbit monoclonal antibody to active-caspase 3 (Santa Cruz) were used to detect the corresponding proteins. Signals were developed with the enhanced chemiluminescence detection system (Pierce).

Statistical analyses

Statistical analysis was performed using SPSS software, version 17.0. Data were analyzed using one-way analysis of variance, and the Tukey HSD test was applied as a post hoc test to determine significance. Statistical significance for two groups was assessed using Student's t-test. The probability level (P) at which differences were considered to be significant was $P<0.05$.

Results

Fan inhibits cell proliferation of MDA-MB-231 cells

To study the effects of Fan on cell proliferation, we treated MDA-MB-231 cells with 5 µM, 10 µM and 30 µM Fan for 1 day to 6 days, and determined cell proliferation by MTT assay. Fan did not affect cell viability at day one (Figure 1). From the second to sixth day, Fan inhibited cell proliferation in a concentration and time dependent manner. Treatment of MDA-MB-231 cells with 10 µM Fan for 6 days and 30 µM Fan for 4 days resulted in 80% of loss of cell viability.

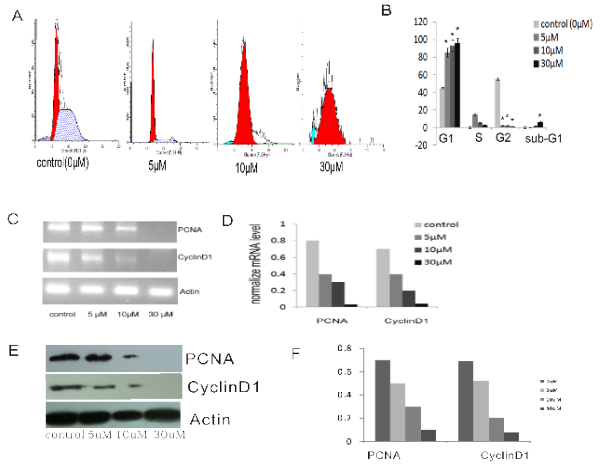

Figure 2. Fan induced Cell Cycle Arrest at G1 Phase in MDA-MB-231 Cells. (A) MDA-MB-231 cells treated with 5 μM, 10 μM and 30 μM Fan for 72 hours were subjected to flow cytometry analysis of the cell cycle distribution. Sub-G1 peak represented apoptosis. (B) Quantification of the dataset from A. (C) MDA-MB-231 cells were treated with 5 μM, 10 μM and 30 μM Fan 48 hours, respectively. RT-PCR analysis was done to determine the mRNA levels of PCNA, cyclin D1 and actin. (D) Quantification of PCNA and Cyclin D1 mRNA levels from RT-PCR as in C. The mRNA levels of PCNA and Cyclin D1 were normalized to beta-actin. (E) Cells were treated as C, total proteins were extracted for the immunoblotting of PCNA, cyclin D1 and actin. (F) Quantification of PCNA and Cyclin D1 levels from immunblots as in E. The protein levels of PCNA and Cyclin D1 were normalized to beta-actin

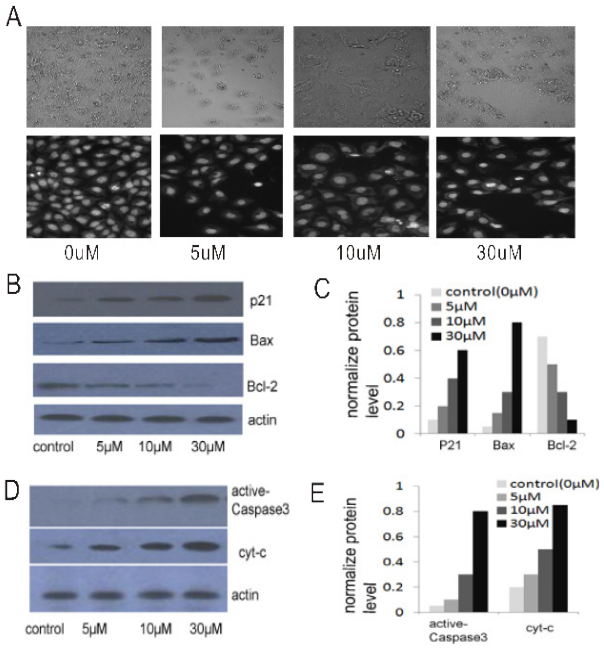

Figure 3. Fan Induced Apoptosis in MDA-MB-231 Cells by Increasing p21, Bax, Cytochrome-c and Active Caspase 3 Protein Level and Inhibiting Bcl-2. (A) Fluorescence microscopy analysis of MDA-MB-231 cells treated with 5 μM, 10 μM and 30 μM Fan for 72 hours. Cells were stained with propidium iodide. The condensed nucleus and cleaved nucleus were enhanced by 10 μM and 30 μM Fan as the arrow indicates. (B) MDA-MB-231 cells were treated with 5 μM, 10 μM and 30 μM of Fan 48 hours, respectively. Western blots were performed to detect the protein levels of p21, Bax, Bcl-2 and actin. (C) Quantification of p21, Bax and Bcl-2 protein levels from immunblots in B. The protein levels of p21, Bax and Bcl-2 were normalized to beta-actin. (D) MDA-MB-231 cells were treated as in B. Western blots were performed to detect the protein levels of cytochrome-c and active–Caspase 3. (E) Quantification of cytochrome-c and active–Caspase 3 levels from immunoblots as in D. The protein levels of cytochrome-c and active–Caspase3 were normalized to beta-actin

Fan arrests cell cycle at G1 phase

To identify the mechanism by which Fan inhibits cell proliferation, we treated MDA-MB-231 cells with 5 μM, 10 μM and 30 μM Fan for 48 hr and analyzed the cell cycle distribution by flow cytometry. Fan significantly arrested cells at G1 phase of the cell cycle and induced a sub-G1 peak, indicating cell apoptosis (Figure 2A). Compared with the control, treatment of cells with 30 μM Fan resulted in 95.99% of cells arrested at G1 phase and 5.93% of cells in sub-G1 (Figure 2B). Thus, Fan prevented cell cycle progression from G1 to S phase transition and induced apoptosis in MDA-MB-231 cells.

G1 phase progression is controlled by cyclin D dependent kinases 4 and 6 (CDK4/6) and PCNA. We asked whether Fan inhibits the activity of CDK4/6. We determined the gene expression of PCNA and cyclin D1, the major regulator of CDK4/6, by examining the mRNA levels of PCNA and cyclin D1 in MDA-MB-231 cells, treated with 5 μM, 10 μM and 30 μM Fan for 48 hours. The mRNA levels of both PCNA and cyclin D1 were significantly reduced by 10 μM Fan and almost ablated by 30 μM Fan (Figures 2C and 2D). Consistent with these findings, we showed that Fan significantly reduced the protein levels of both PCNA and cyclin D1 (Figures 2E and 2F). To gain further insight into the molecular mechanisms by which Fan arrested cell cycle at the G1 phase, we detected p21 protein levels and found that Fan increased p21 in a concentration-dependent way (Figures 3B and 3C). The results indicated that Fan inhibited cell proliferation by down-regulating PCNA gene expression and prevented G1 to S phase progression through blocking cyclin D1 expression.

Fan increases the ratio of Bax/Bcl-2 and the activation of Caspase 3 and cytochrome-c

To characterize the induction of apoptosis by Fan (Figure 2B), we treated MDA-MB-231 cells with 5 μM, 10 μM and 30 μM Fan for 72 hours, stained the cells with PI and observed cell morphology under microscope. The cell nucleus became condensed when treated with 5 μM Fan for 72 hours. Moreover, 10 μM and 30 μM Fan for 72 hours induced apparent apoptotic bodies and nuclear cleavage (Figure 3A). To confirm our findings, we determined the protein levels of Bax and Bcl-2 by immunoblotting. Bax protein was gradually induced with the increase in dosage of Fan. In contrast, Bcl-2 was significantly down-regulated as the concentration of Fan increased (Figure 3B), indicating that Fan induces cell apoptosis by increasing the ratio of Bax/Bcl-2(Figure 3C). To corroborate these observations, we further detected activated-Caspase 3 and cytochrome-c in MDA-MB-231 cells treated with Fan. Interestingly, Fan significantly increased the activated-Caspase 3 and cytochrome-c

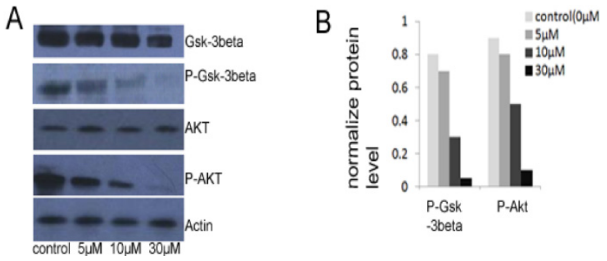

Figure 4. Fan Reduced the Activity of the AKT/GSK-3beta Pathway. (A) MDA-MB-231 cells were treated with 5 μM, 10 μM and 30 μM Fan for 48 hours, respectively. Total proteins were extracted for the immunoblotting of AKT, p-AKT, GSK-3beta, p-GSK-3beta and actin. (B) Quantification of p-AKT and p-GSK-3beta levels from immunoblots in A. The protein levels of p-AKT and p-GSK-3beta were normalized to beta-actin

protein levels in a dose dependent manner (Figures 3D and 3E).

Fan inhibits activation of the AKT/GSK-3beta pathway

Arrest of cells in the G1 phase of the cell cycle provides a window for the induction of cell apoptosis. To determine whether Fan inhibits cell proliferation via down-regulation of the AKT/GSK pathway, we examined the expression of AKT, GSK-3beta, p-AKT and p-GSK-3beta in MDA-MB-231 cells treated with Fan. Fan did not affect the total protein levels of AKT and GSK-3beta, but did dramatically decrease p-AKT or p-GSK-3beta levels (Figure 4A). The p-AKT and p-GSK-3beta signals were 10 times and 16 times lower in cells treated with 30 μM Fan than levels observed in the control cells (Figure 4B), indicating that Fan blocks cell proliferation via inhibition of the AKT/GSK-3beta pathway.

Discussion

In this study, we believe that this is the first time that it has been shown that Fan inhibited cell proliferation and induced cell apoptosis in the breast cancer MDA-MB-231 cell line. This was accompanied by downregulation of AKT/GSK-3beta signaling. Many new therapeutic strategies have been explored for the treatment of breast cancer. For example, the semi-synthetic epothilone B derivative, ixabepilone, has been demonstrated as having potent anti-tumor activity against breast cancer cells (Boehnke et al., 2009). Hedgehog (Hh) signaling has been utilized as a therapeutic target for patients with receptor-α negative breast cancer (Kameda et al., 2009). Targeting of Notch signaling pathway with drugs such as the γ-secretase inhibitors also showed a promising future development for the treatment of breast cancer (AI-hussaini et al., 2011). In this study, we found that Fan down-regulated the AKT pathway, one of the key cancer cell growth-promoting signaling pathways. Moreover, Fan induced MDA-MB-231 cell apoptosis by increasing the ratio of Bax/Bcl-2 and the levels of active caspase 3 and cytochrome-c. Thus, we propose that Fan is a promising therapeutic agent for the treatment of breast cancer. Our data demonstrated that Fan inhibits the proliferation of breast cancer MDA-MB-231 cells. Self-sufficiency in cell growth and resistance to anti-proliferative signaling are hallmarks of cancer cells. To ensure homeostasis of the cell number and maintenance of normal tissue architecture and function, normal tissues tightly control the production and release of growth-promoting signals, which instruct entry to and progression through the cell growth and division cycle. Cancer cell has obtained the capability of deregulating these signal pathways that regulate progression through the cell cycle as well as cell growth. In our experiment, Fan inhibited the growth of MDA-MB-231 cells in a concentration and time dependent manner (Figure 1). FACS analysis demonstrated that Fan arrested MDA-MB-231 cell cycle at G1 phase (Figure 2A and 2B). PCNA is one of the most important factors governing cell proliferation. Fan significantly reduced both the mRNA and protein levels of PCNA. Cell cycle is controlled by cyclin dependent kinases. Cyclin D1 is the major G1 phase cyclin and overexpressed in most cancer cells. We found that cyclin D1 mRNA and protein levels were significantly down-regulated by Fan. AKT-Gsk-3beta signaling promotes cell cycle progression by upregulating the positive regulators of cell growth including PCNA and cyclin D1 (Hardy et al., 2000; Maffucci et al., 2007). In agreement with the reduction of PCNA and cyclin D1, Fan dramatically inhibited the activation of the AKT/GSK-3beta signaling pathway in MDA-MB-231 cells. Our data suggest that Fan inhibits MDA-MB-231 cell proliferation via the AKT/GSK-3beta-cyclinD1 signal pathway.

In addition, we found that Fan induced cell apoptosis in MDA-MB-231 cells as evidenced by nuclear condensation. The ratio of Bax/Bcl-2 and active caspase 3 and cytochrome- c protein are critical factors for the induction of apoptosis (Katiyar et al., 2005; Mantena et al., 2006; Jackel et al., 2011). Consistent with these findings, Fan increased the ratio of Bax/Bcl-2 and up-regulated the protein levels of active caspase 3 and cytochrome-c.

In conclusion, our study demonstrated that Fan inhibited cell proliferation in MDA-MB-231 cells through suppression of the AKT/Gsk-3beta/cyclinD1 signaling pathway. Furthermore, Fan induced apoptosis by increasing the ratio of Bax/Bcl-2 and active caspase 3 and cytochrome-c, suggesting that Fan is a potential drug for the therapy of breast cancer.

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