Fangchinoline Induced G1/S Arrest by Modulating Expression of p27, PCNA, and Cyclin D in Human Prostate Carcinoma Cancer PC3 Cells and Tumor Xenograft

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Prostate cancer (PCA) is the most common invasive malignancy and the second leading cause of cancer-related death in males. The present study investigated the effects of fangchinoline (Fan), an important compound in Stephania Tetradra S. Moore (Fenfangji) with pain-relieving, blood pressure-depressing, and anti-bacterial activities, on human PCA. It was found that Fan inhibited human prostate cancer cell lines (PC3) cell proliferation in a dose- and time-dependent manner. Studies of cell-cycle progression showed that the anti-proliferative effect of Fan was associated with an increase in the G1/S phase of PC3 cells, Western blot results indicated that Fan-induced G1/S phase arrest was mediated through inhibition of cyclin-regulated signaling pathways. Fan induced p27 expression and inhibited cyclin D and proliferating cell nuclear antigen (PCNA) expression in PC3 cells. Increased exposure time to Fan caused apoptosis of PC3 cells, which was associated with up-regulation of pro-apoptotic proteins Bax and caspase 3, and down-regulation of anti-apoptotic protein Bcl-2. Furthermore, Fan had anti-tumorigenic activity in vivo, including reduction of tumor volume and pro-apoptotic and anti-proliferative effects in a PC3 nude mouse xenograft. Taking all this together, it can be concluded that Fan is an effective anti-proliferative agent that modulates cell growth regulators in prostate cancer cells.

Key words: prostate cancer; fangchinoline; human prostate cancer cell lines (PC3); tumor xenograft; apoptosis

Prostate cancer (PCA) is the most common type of cancer and the leading cause of cancer death among men in the United States. It is estimated that in 2008, approximately 186,320 new cases and 28,660 prostate cancer-related deaths will occur in the United States.† Radial prostatectomy and radiation therapy are potentially curative. However, over one-third of patients will develop metastases for which the current treatment of choice is androgen ablation therapy. Unfortunately, the majority of diseases eventually progress to a hormonal refractory prostate cancer (HRPC). The current treatment modality for those patients is chemotherapy based on docetaxel, which provides only marginal improvements in survival for patients with HRPC.‡ Therefore, novel experimental therapeutics should be investigated to overcome the poor efficacy of current treatment modalities.

Contemporary research has proven that many herbal extracts and isolated compounds possess anti-tumor activities. Clinical trials on the use of herbal medicinal agents have been conducted in order to establish novel chemotherapeutic avenues. It is known that Fan, an important compound in Stephania Tetradra S. Moore (Fenfangji), can inhibit the antinociception potency of morphine,§ and it is also known to lower blood pressure as a non-specific calcium channel antagonist.¶

In recent research, tetrandrine (Tet), a derivative of Fan, was found to be a potential anti-tumor agent. First, Tet elicits in vivo tumor suppressive effects on mouse ascites tumors.¶¶ Tet mediates the responses of cell proliferation and cytotoxicity by blocking the voltage-gated Ca2+ channel, intracellular Ca2+ pumps, and large-conductance Ca2+-activated K+ (BK) channels.¶¶ It is reported that Tet-mediated expression and activation of different proteins is involved in apoptotic cell death and that Tet-induced apoptosis in HepG2 cells is associated primarily with the mitochondrial pathway involving cytochrome c release and caspase 9 activation, which is dependent on the activation of downstream caspase 3 and 8.¶¶ It is also reported that Tet induces HT29 human colon carcinoma cells apoptosis by down-regulating key G1 regulatory proteins, including CDK 4 and CDK 6 and that it induces p53 and A549 human lung carcinoma cell apoptosis by inducing cdk inhibitor p21 and cyclin D1 and activating caspase 3.¶¶ Tet is also

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Abbreviations: BK, Ca2+-activated K+ channels; DMEM, Dulbecco’s modified essential medium; DMSO, dimethyl sulfoxide; Fan, fangchinoline; FBS, fetal bovine serum; Fenfangji, Stephania Tetradra S. Moore; HRPC, hormonal refractory prostate cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered solution; PCA, prostate cancer; PCNA, proliferating cell nuclear antigen; PC3, human prostate cancer cell lines; RT-PCR, reverse transcription-polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tet, tetrandrine
known to be a promising candidate for combination with chemotherapeutic agents.11)

However, there is little research on Fan as an anti-tumor agent, while Tet has proven to be a potential anti-cancer agent. Several epigenetic alterations that lead to constitutively active mitogenic and cell-signal survival pathways, as well as loss of the apoptotic response, are involved in uncontrolled growth of PCA, leading to androgen-independent growth, apoptosis resistance, and increased expression and secretion of angiogenic factors.12) Hence, one targeted approach for PCA prevention, growth control, or treatment can be the inhibition of molecular events involved in PCA growth, progression, and apoptosis resistance. In light of the above, we examined the anti-proliferative apoptotic effects of Fan on androgen-independent PC3 cells. Next, we employed Western blots to gain further insight into protein expression patterns that might play a role in the regulation of these cellular events. Finally, we examined the growth-inhibitory effects of Fan in vivo using a nude mouse xenograft.

Materials and Methods

Materials. Fan was obtained from China Chengdu Scholar BioTech (Chengdu, China). Antibody, Anti-actin (AC-74), was from Beyotime Institute of Biotechnology (Jiangsu, China), and anti-active caspase 3, anti-Bcl-2, anti-Bax, anti-PCNA, and anti-p27 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. Human prostatic carcinoma PC3 cell lines were obtained from the China Center for Type Culture Collection (Wuhan University, Wuhan, China) and cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Cell proliferation was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). PC3 cells (1 × 10⁴ cells/ml) were cultured in 96-well plates and treated with Fan for 1, 2, 3, and 4 d at various concentrations (10–30 μM). After the addition of MTT solution, the cells were incubated at 37 °C for 4 h. Crystallized MTT was dissolved in dimethyl sulfoxide (DMSO) (Sigma, New York, CA), and the absorbance of the resulting formazan was measured spectrophotometrically at 492 nm (Sunrise, Tecan, Switzerland).

Fluorescent microscopical examination. PC3 cells (2 × 10⁴ cells/ml) were seeded in a 24-well tissue culture plate and treated with 30 μM Fan for 24 h. After treatment, the cells were fixed in 75% v/v ethanol for 1 h at 4 °C. They were washed once with phosphate buffered solution (PBS) and resuspended in cold propidium iodide staining buffer (10 μg/ml) in PBS (pH 7.4) for 30 min in the dark. The nuclear morphology of the cells was visualized with an Olympus IX70 microscope (Olympus, Pennsylvania, CA). Photographs were taken using Winview32 (Princeton Instruments, Florida, CA).

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Table 1. RT-PCR Primers Used in Each Gene Fragment Amplification

<table>
<thead>
<tr>
<th>Primers’ names</th>
<th>Sequences (5’–3’)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>actin(NM_001100)</td>
<td>5’caggctacatgctcgca3’;</td>
<td>432</td>
</tr>
<tr>
<td>PCNA(NM_002592)</td>
<td>5’caaaaaggagtttaacggc3’;</td>
<td>410</td>
</tr>
<tr>
<td>cyclinD1(NM_053056)</td>
<td>5’gctactcagcttcatcga3’;</td>
<td>310</td>
</tr>
</tbody>
</table>

RNA extraction and semiquantitative RT-PCR. Total RNA was extracted and DNase was used to eliminate contamination by genomic DNA. The reverse transcription-polymerase chain reaction (RT-PCR) primers used for gene amplification are listed in Table 1. After denaturation at 94 °C for 2 min, polymerase chain reaction was carried out in a DNA thermal cycler (Biomerieux Thermocycler, Göttingen, Germany) for 30 cycles. Each cycle included denaturation at 94 °C for 40 s, annealing at 54 °C for 40 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The PCR products were run on 2% agarose gel in TAE buffer (40 mmol/l Tris acetate and 1 mmol/l ethylenediaminetetraacetic acid), and visualised by ethidium bromide staining using Gel-doc Image (Bio-Rad laboratories, California, CA).

Fluorescence-activated cell sorter analysis. PC3 cells (2 × 10⁵) were trypsinized and washed with PBS after treatment with Fan at a concentration of 30 μM for 1 d. Cells (1 × 10⁶) were fixed with 75% ethanol at −20 °C overnight. The cells were stained with propidium iodide and RNAase at 37 °C. Analysis of samples was done using a Becton Dickinson Facstar flow cytometer and Becton Dickinson cell fit software (BD Biosciences, San Jose, CA).

Western blotting. PC3 cells (1 × 10⁶) were treated with various concentrations of Fan (10, 20, and 30 μM) for 24 h. Proteins were obtained by lysing in ice-cold PBS buffer for 30 min on ice. Total cell proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and probed with the following antibodies: active caspase 3, Bax, and Bcl-2. Immunoblots were developed and visualized by the enhanced chemiluminescence detection system (super signal west pico chemiluminescent substrate, Pierce Biotechnology, Rockford, CA). Each blot was stripped and reprobed with β-actin antibody as an internal control. Densitometric analysis was done by Quantity One version 4.1.1 Basic (Bio-Rad).

Tumor xenographs in nude mice. The experimental procedures were approved by our institutional animal research ethics committee with reference to the Chinese Community guidelines for the use of experimental animals. Six-week-old male Balb/c-nu-nu mice were obtained from the Laboratory Animal center of Hebei Province, China. The experimental licence number is scxk (Hubei) 2003-0005. The mice were kept under sterile conditions and pathogen-free in isolated pathogen-free ventilation chambers at 20–22 °C and 45–50% relative humidity. The animal-rearing facility was maintained on a 12 h light-dark cycle. All the animals were given free access to sterilized food and water, and were under habituation for 10 d before experimentation.

Cell suspension was obtained by trypsinization of confluent PC3 cells. The mice were randomly assigned to various treatment groups of 12 mice each, and anesthetized with i.p. injection of 75 mg/kg ketamine and 10 mg/kg xylazine. The mice were treated with intravenous injection of Fan once daily (days 0–16).Suspension cells were injected subcutaneously into the back of each animal at a cell density of 1 × 10⁶ in 200 μl of phosphate-buffered saline. The day of tumor implantation was designated day 0. Tumors became palpable 3 d after xenografting. Tumor volume was measured using a digital caliper every 4 d, and was calculated as (length × width²)/2. The mice were monitored for 16 d after tumor inoculation. The body weights of all animals were recorded throughout the entire experimental period to assess drug toxicity. Any mortality during the course of the study was also recorded.
Immunohistochemical analysis of cell proliferation and apoptosis in tumor tissues. On day 16, all the animals were killed by i.p. injection of an overdose (100 mg/kg) of ketamine, followed by cervical dislocation. Excised tumor specimens were fixed for paraffin embedment. Sections of tumor samples 4 μm in thickness were prepared on slides. The slides with sample sections were blocked with 10% normal goat serum to minimize non-specific background, and were incubated overnight with anti-PCNA, p27 monoclonal antibody at 4 °C. The positively stained cells were visualized by incubating the sections with 3,3-diaminobenzidine and Mayer’s hematoxylin counterstain. A negative control was prepared by similar procedures but without adding the primary antibody.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 software. Student’s t-test was used to compare the mean values of the two groups. The data for three or more groups were compared by one-way analysis of variance, followed by Dunnett’s post hoc test. Final values are expressed as mean ± SEM. A difference of at least p < 0.05 was considered statistically significant.

Results

Anti-proliferative effects of Fan in PC3 cells
The results of our study indicate the effect of Fan in ameliorating cancer cell growth in a dose- and time-dependent manner. Fan incubation for 3 d significantly reduced the proliferation of PC3 cells, by 63% and 86%, at the two higher concentrations of 20 and 30 μm respectively (Fig. 1A).

One of the early events of apoptosis is the condensation of nuclear chromatin. Hence we investigated the morphology of Fan-treated cells using propidium iodide staining. Cells treated with 30 μm Fan for 24 h displayed the typical morphology of nuclear chromatin condensation as compared with the control cells after Fan treatment (Fig. 1B).

Fan caused cell-cycle arrest at the G1/S phase in PC3 cells
Cells appeared to accumulate at the G1 phase following Fan treatment, with a concomitant decrease in the percentage of cells in the S phase. The accumulation of cells in the G1/S phase began to occur following 1 d of drug treatment (Fig. 2A).

The transcriptional levels of cyclin D and PCNA were analyzed by RT-PCR from Fan-treated PC3 cells. It was found that the transcriptional level of cyclin D was decreased by Fan at 30 μm as compared with that of the control. Furthermore, the transcriptional level of PCNA was decreased by Fan at 30 μm as compared with that of the control (Fig. 2B).

Fan-induced apoptosis in PC3 cells
We assessed the protein expression of active caspase 3, Bcl-2, and Bax in PC3 cells by western blotting. As shown in Fig. 2C, expression of active caspase 3 in PC3 cells increased following 24 h of Fan treatment at various concentrations, of 10, 20, and 30 μm. These findings indicate that Fan-induced apoptosis is caspase-dependent. Furthermore, it was found that the expression of the pro-apoptotic protein Bax increased following 24 h of Fan treatment at concentrations, of 10, 20, and 30 μm, and a decrease in the expression of anti-apoptotic protein Bcl-2 was also induced at a concentration of 30 μm (Fig. 2D).

The anti-tumorigenic effects of Fan in a nude mouse xenograft
The anti-tumorigenic effect of Fan on PCA was further illustrated in vivo in a nude mouse xenograft. On the day of sacrifice (day 16), Fan treatments at the given doses resulted in about 57.8% tumor suppression, and Fan-induced tumor volume reduction was significant from day 8 onwards, with average suppression of 30% (Table 2, Fig. 3A).

The levels of mRNA and protein extracted from Fan-treated and non-treated tissues of tumor were analyzed through RT-PCR and western blotting. It was shown clearly that there were decreases in the mRNA levels of cyclin D and PCNA in PC3 cells treated with Fan (Fig. 3B). Besides, the proportion of active caspase 3, p27, and Bax increased in the PC3 cells treated with Fan, although the levels of Bcl-2 protein decreased in the treated cells (Fig. 3C).

Immunohistochemical assessment of cell proliferation in the tumor sections showed that Fan treatments reduced the numbers of visible PCNA-labeled cells. It was also found that the numbers of p27-labeled cells increased under treatment with Fan (Fig. 4).
Discussion

The molecular biology of PCA and its progression is characterized by the aberrant activity of several regulatory pathways, both within the prostate cells and in surrounding tissues. These pathways can be broadly grouped into apoptosis, androgen receptor signaling, signal transduction, cell cycle regulation, cell adhesion and cohesion, and angiogenesis. Variations at the DNA, RNA, or protein levels of the molecules involved in these pathways are all potential candidate markers of prognosis and therapeutic response. The control of cell-cycle progression in cancer cells is considered an effective strategy to prevent or delay tumor growth. This assumption is based on a molecular analysis of human cancer, wherein cell cycle regulators are frequently de-regulated in most of the common malignancies.

Despite recent advancement in understanding the carcinogenic process of PCA, the increasing incidence and relatively low remission rate of chemotherapy have prompted the scientific community to establish more effective treatment regimens by adopting novel and innovative approaches. The discovery and use of active medicinal compounds from herbal and natural sources has provided alternative treatment choices for patients.
This is the first report that clearly characterizes the anti-tumor properties of Fan in prostate cancer cells and tumor xenograft.

In order to identify the mechanism involved in Fan-induced growth suppression, we first investigated the events that lead to inhibition of prostate cancer cell proliferation. In response to drug-induced inhibition of cancer-cell growth, mammalian cells activate different cell-cycle check points.22) We had found that Fan causes PC3 cells to accumulate in the G1/S phase. The major regulator of the G1 to S transition is the cyclin D-CDKs complexes.23–25) The complexes are activated at different points after certain intervals during the cell cycle, and can also be regulated by several exogenous factors. Cyclin D/CDKs complexes are held in an inactive state by phosphorylation of CDKs at negative regulator sites, such as Thr-160 in CDK2 and Thr-172 in CDK4.23) De-phosphorylation of the negative regulatory sites is needed to activate the CDKs/cyclin D complex, and CDK activity is additionally regulated by p27 proteins.23) Hence, we studied the involvement of Fan in

**Table 2.** Effect of Fan Treatment on Tumor Volume (mm$^3$) of A Nude Mouse Xenograft

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 12</th>
<th>Day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (male)</td>
<td>112 ± 16</td>
<td>202.5 ± 20</td>
<td>378 ± 18</td>
<td>450 ± 18</td>
</tr>
<tr>
<td>Fan (5 mg/kg)</td>
<td>56 ± 8</td>
<td>141.75 ± 20$^a$</td>
<td>187.5 ± 12.5$^a$</td>
<td>190 ± 60$^a$</td>
</tr>
</tbody>
</table>

* $p < 0.05$ versus control.

**Fig. 3.** Anti-Tumor Effects of Fan in PC3 Xenograft.

A, Photographic illustration of tumors from control and Fan-treated nude mice on the day of sacrifice (day 16). B, mRNA levels of cyclin D, PCNA, and actin were isolated from tumor tissues in both the control and the drug treatment group. mRNA was detected by the RT-PCR method. C, we also assessed the protein expression of active caspase 3, p27, Bcl-2, and Bax in tumor tissues in both the control and the Fan treatment group by western blotting. Each point represents the mean ± SEM for three independent experiments. *$p < 0.05$ versus control.

**Fig. 4.** Immunohistochemical Analysis in the PC3 Xenograft.

Representative images of the microscopic images obtained from the control and the Fan treatment groups of subsequent HE, PCNA, and p27 staining and Mayer’s hematoxylin counterstaining are shown.
p27-modulated cell-cycle regulatory events that are operational in the G1/S phase as a mechanism of cell-cycle dysregulation in human prostate cancer cells. The results of the present study show an increase in the expression of p27 proteins, which inhibit formation of the CDKs/cyclin D complex.

PCNA was long regarded only as a proliferation marker because it is expressed late in the G1 phase and early in the S phase. Recent evidence indicates a much broader range of functions in which PCNA is involved. It is a homotrimer protein that binds to a variety of factors required for cell-cycle progression, replication, and DNA repair. The result described here that PCNA is downregulated by Fan, intimately links PCNA to the cell-cycle regulating machinery in PC3 cells. Furthermore, the results from the studies in vivo also indicate that Fan inhibited cell growth and induced G1/S phase cell cycle arrest.

We also identified the mechanism of reduction in cell viability and induction of programmed cell death in PC3 cells by Fan. Bcl-2 family proteins have emerged as critical regulators of mitochondria-mediated apoptosis by functioning as either promoters (e.g., Bax) or inhibitors (e.g., Bcl-2) of the cell-death process. The increase in Fan-induced apoptosis was associated with an increase in the levels of Bax, which heterodimerizes with and thereby inhibits Bcl-2. Our results indicate that Fan regulates Bcl-2 and Bax expression and thus leads to the induction of apoptosis. Furthermore, the observation of caspase 3 activation also confirms that the promotion of apoptosis by Fan involves a caspase-dependent pathway. Apoptosis plays a crucial role in eliminating mutated neoplastic and hyperproliferating neoplastic cells from the system, and hence is considered to be a protective mechanism against the development of cancer.

To test the physiological relevance of in vitro Fan-mediated anti-tumor effects in vivo, the anti-tumor effects of Fan were evaluated in PC3 xenografted nude mice. Fan significantly inhibited tumor growth of PC3 cells xenografted into the mice without causing mortality, significant weight loss, or other major side effects. These observations are in agreement with our in vitro studies showing that treatment of PC3 cells with Fan results in concentration-dependent induction of apoptosis. The promising anti-tumor activity coupled and lack of toxicity of Fan suggests that it has the potential to be a novel therapeutic agent in PCA chemotherapy. Moreover, the present study explains the in vivo Fan-mediated suppression of Bcl-2 and increase in Bax and active caspase 3. Thus monitoring the expression levels of apoptotic regulatory molecules affected by Fan may be an alternative strategy for monitoring its effect in the primary stages of PCA treatment. Based on the above findings, it is reasonable to conclude that apoptotic induction is an essential event in the Fan-mediated suppression of PC3 cell growth in vivo. However, our observations should be confirmed in further animal studies to establish more definitely the in vivo relevance of the in vitro findings. Moreover, we used relatively high Fan doses in treating the mice. If Fan can be administered into tumor feeding vessels, higher concentrations can be achieved with lower doses. Further dosing studies are also needed if we are to extrapolate our findings to humans.

In summary, we found in this study that Fan from Fenfangji inhibited human PC3 cell growth both in vitro and in vivo. This suggests the possibility of developing Fan further as an alternative treatment option in PCA therapy.

Acknowledgments

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References