

## Apoptotic Effects of 6-Gingerol in LNCaP Human Prostate Cancer Cells

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**Objective:** 6-Gingerol, one component of ginger (*Zingiber officinale*) compound, has been known to possess anti-inflammatory, analgesic, anti-emetic, and anti-cancer effects. In this study, the apoptotic ability of 6-gingerol was investigated in human prostate cancer cells.

**Methods:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, flow cytometry, and western blot analysis were done in LNCaP human prostate cancer cell lines treated with the various doses of 6-gingerol for the different durations of drug exposure.

**Results:** 6-Gingerol in doses ranging from 100 to 300  $\mu$ M induced dose- and time-dependent inhibition of cell viability in prostate cancer cells by using MTT assay. Maximal inhibition of cell viability was observed at 300  $\mu$ M of 6-gingerol for 48 hours treatment in LNCaP cells. 6-Gingerol at the dose of 100  $\mu$ M did not produce any significant change in apoptotic cells in flow cytometry analysis. However, significant increase in sub-G0/G1 phase was observed in cells treated with 200 and 300  $\mu$ M of 6-gingerol. Any significant cell cycle arrest was not induced by 6-gingerol. In western blotting analysis, expression of caspase-3 was not evident in cells treated with 6-gingerol for 24 hours. However, 48 hours treatment with 6-gingerol altered the expression of caspase-3 in LNCaP cells. Expression of cleaved poly showed the dose-dependent fashion in both 24 hours and 48 hours treatment of 6-gingerol.

**Conclusion:** These observations suggest that 6-gingerol may induce apoptosis in LNCaP human prostate cancer cells.

**Keywords:** 6-Gingerol; Apoptosis; Caspase-3; Poly(ADP-ribose) Polymerases; Human prostate cancer cells

### INTRODUCTION

Ginger (*Zingiber officinale*) has been utilized world wide as a spice and a traditional herb to relieve headache, nausea, cold, and etc. [1,2]. It contains pungent phenolic substances collectively known as gingerol, shogaol, and zingerone [3]. Of these, 1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone (6-gingerol) (Fig. 1) has been shown to have anti-oxidant [4], anti-inflammatory [5], and anti-tumor effects [6-8].

Regarding the effect of 6-gingerol in various cancer cell models, 6-gingerol has been reported to produce anti-tumor effects. 6-gingerol inhibited pulmonary metastasis in mice bearing B16F10 cells through the activation of CD8 T cells [9] and inhibited the tumor growth of skin tumor in ICR mouse [10]. 6-Gingerol caused anti-cancer effects also in gastric cancer cells [11], pancreatic cancer cells

[12], and colorectal cancer cells [13].

Prostate cancer is the second most prevalent malignancy and second leading cause of cancer-related deaths among men in the USA [14]. In Korean male cancers, prostate cancer is also the fifth leading one in its incidence and mortality during 2008 in Korea [15]. Recently, chemoprevention and intervention strategies using anticancer agents are suggested as promising alternative options [16,17].

However, the effect of 6-gingerol on the cell growth and death in human prostate cancer cells has not been well elucidated. The present study therefore attempted to determine whether 6-gingerol has anti-cancer activities by observing the *in vitro* effect of 6-gingerol in LNCaP human prostate cancer cells and to elucidate its molecular mechanisms for 6-gingerol-induced responses.

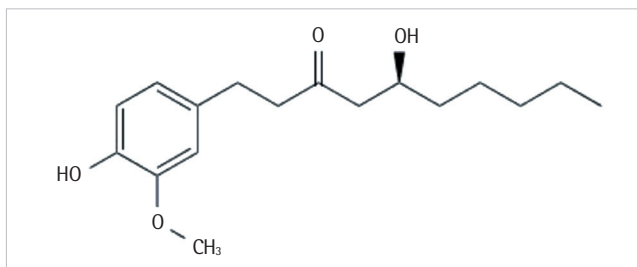
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**Fig. 1.** Structure of 6-gingerol.

## MATERIALS AND METHODS

### 1. Chemicals and Cell Culture

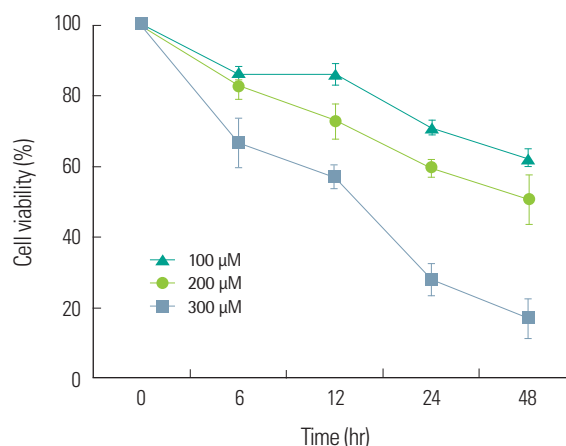
6-Gingerol was purchased from Dalton Pharma Services (Toronto, ON, Canada). It was dissolved in 0.1% ethanol. Human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 media supplemented with 5% fetal bovine serum, penicillin (100 kU/L), streptomycin (100 mg/L), and 2 mM of glutamine at 37°C in a 5% CO<sub>2</sub> incubator.

### 2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) Assay for Cellular Viability

The viability of the cells was assessed by MTT assay which is based on the reduction of MTT through the mitochondrial succinate dehydrogenase of intact cells into a purple formazan product. One mL aliquot of the exponentially growing LNCaP cells containing  $1 \times 10^5$  cells/mL was added to each well of 24-well plates and incubated with various amounts of the 6-gingerol. MTT solution was added and incubated for 2 hours at 37°C in a 5% CO<sub>2</sub> incubator. The resulting MTT-formazan product was dissolved by the same volume of lysis buffer and the incubation was continued overnight at 37°C. The amount of formazan was determined by measuring the absorbance at 570 nm using an ELISA plate reader (Bio-Rad 550; Bio-Rad Laboratories Inc., Hercules, CA, USA). The assay was done three times independently.

### 3. DNA Cell Cycle Analysis by Flow Cytometry

Flow cytometric DNA analysis was performed by the available method with minor modifications. Growing LNCaP cells at a density of  $1 \times 10^5$  cells/mL were untreated or treated with 6-gingerol. The cells were then prepared as a single cell suspension in phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol, and maintained at 4°C overnight. The cells were harvested by  $500 \times g$



**Fig. 2.** Effect of 6-gingerol on the cell viability of LNCaP human prostate cancer cells. Cells were treated with the dose ranging from 100 through 300 μM of 6-gingerol for 6, 12, 24, and 48 hours. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Each value represents the mean  $\pm$  SD of three independent experiments.

centrifugation for 10 minutes, resuspended in PBS supplemented with 0.1% Triton-X 100 and DNase free RNase (100 g/mL), incubated at 37°C for 30 minutes, and stained with propidium iodide in the dark at 4°C for 30 minutes. The red fluorescence of the individual cell was determined with the flow cytometry (BD FACScalibur flow cytometer, BD Biosciences, San Jose, CA, USA).

### 4. Western Blot Analysis

Equal amount of proteins (20 to 50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the gel was blotted onto a polyvinylidene fluoride membrane. The blotted membranes were first blocked with tris-buffered saline containing 5% nonfat dried milk for 2 hours at room temperature and followed by probing with primary antibodies against proteins-of-interest in 3% nonfat dried milk at 4°C for overnight. Membranes were then washed three times with tris-buffered saline Tween-20 (TBST) and incubated with secondary antibodies for 2 hours. Membranes were further washed three times with TBST and visualized using electrochemiluminescence detection system by LAS-3000 luminescent image analyzer (Fuji Photo Film, Tokyo, Japan). The primary antibodies against caspase-3 and poly ADP-ribose polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA, USA). The acquisition and analysis of samples were done three times independently.

### 5. Statistical Analysis

Data were presented with mean  $\pm$  SD. Statistical analysis was

**Table 1.** Effect of 6-gingerol on cell cycle phase distribution of LNCaP human pancreatic cancer cells

Treatment	% cells			
	Sub-G0/G1	G0/G1	S	G2/M
Control	16.28±0.29	50.93±2.58	11.82±2.13	20.15±0.65
100 µM 24 hr	16.54±0.94	49.96±1.55	11.94±0.50	21.48±5.13
200 µM 24 hr	24.10±2.53*	48.70±0.05	9.57±0.98	17.36±3.35
300 µM 24 hr	23.09±2.25*	51.01±1.36	9.90±1.06	15.94±2.75
100 µM 48 hr	15.87±1.76	49.57±1.00	11.23±2.53	22.99±4.22
200 µM 48 hr	31.83±4.18*	45.61±2.62	8.16±1.97	14.27±3.81
300 µM 48 hr	29.46±2.91*	48.23±3.92	8.97±0.57	13.15±4.19

Values are presented as mean ± SD.

Cells were treated with 100, 200, and 300 µM of 6-gingerol for 24 and 48 hours. These cells were then harvested, washed in phosphate-buffered-saline, fixed with 70% ethanol, and stained with propidium iodide. DNA contents were then analyzed by flow cytometry. All phases are represented in percentage.

\*P < 0.05 vs. control.

done using Student's t-test and ANOVA test. Significance was defined as P < 0.05.

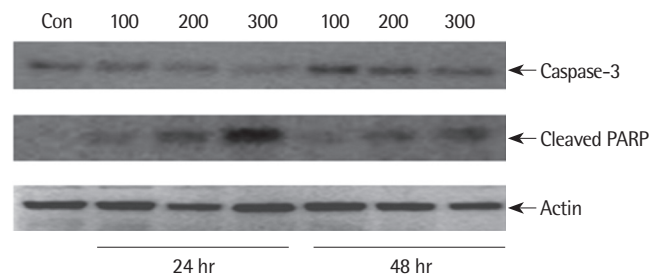
## RESULTS

### 1. Effect of 6-gingerol on Cell Viability of LNCaP Human Prostate Cancer Cells

We evaluated the effects of 6-gingerol on cell viability in human prostate cancer cells (Fig. 2). These cells were treated with the range of 100, 200, and 300 µM of 6-gingerol for 6, 12, 24, and 48 hours. The viability was determined by MTT assay. 6-Gingerol (100-300 µM) produced a dose- and time-dependent inhibition of cell viability in human prostate cancer cells. When cells were treated for 24 hours, 6-gingerol inhibited 29.9% (100 µM), 40.6% (200 µM), and 72% (300 µM) of prostate cancer cell growth. Maximal inhibition of cell viability was observed at 300 µM of 6-gingerol for 48 hours treatment in LNCaP cells.

### 2. Effect of 6-gingerol on Cell Cycle Phase Distribution in LNCaP Cells

In order to elucidate the mechanism for 6-gingerol-induced inhibition of cell viability, we carried out flow cytometry analysis on LNCaP cells treated with 100, 200, and 300 µM of 6-gingerol for 24 hours and 48 hours (Table 1). 6-gingerol at the dose of 100 µM did not produce any significant change in apoptotic cells. However, the significant increase in sub-G0/G1 phase was observed in cells treated with 200 and 300 µM of 6-gingerol, compared to the control. In the identical fashion for both doses of 6-gingerol, 48



**Fig. 3.** Effect of 6-gingerol on the expression of caspase-3 and poly ADP-ribose polymerase (PARP) cleavage in LNCaP cells. Western blot analysis was done in LNCaP cells treated with 100, 200, and 300 µM of 6-gingerol for 24 and 48 hours. Results are representative of three independent experiments. Actin was used as a standard for each sample.

hours treatment showed a little higher distribution of cells in sub-G0/G1 phase than 24 hours treatment did, suggesting a time-dependent manner. 6-Gingerol did not produce any significant cell cycle arrest. 6-Gingerol-induced increase of apoptotic cells was consistent with the inhibitory cell viability effect by 6-gingerol in LNCaP cells. These results suggest that apoptosis is mainly responsible for the inhibition of cell viability by 6-gingerol in LNCaP prostate cancer cells.

### 3. Effect of 6-gingerol on the Expression of Apoptosis-Related Caspase-3 in LNCaP Cells

Caspases are responsible for many of the biochemical and morphological changes during apoptosis. To investigate the molecular pathways for 6-gingerol-induced apoptosis in prostate cancer cells treated with 100, 200, and 300 µM of 6-gingerol for 24 and 48 hours, we performed the western blotting analysis of apoptotic protein, caspase-3. In 24 hours treatment of 6-gingerol, expression of caspase-3 was not evident. However, 6-gingerol in 48 hours treatment induced the expression of caspase-3 in LNCaP cells (Fig. 3).

### 4. Effect of 6-gingerol on the Expression of Cleaved PARP in LNCaP Cells

Since PARP-specific cleavage follows the activation of caspase-3 in apoptotic pathways, we conducted the western blotting experiment in LNCaP cells treated with 100, 200, and 300 µM of 6-gingerol for 24 and 48 hours using the antibody against PARP. Cleaved PARP expression was observed in both 24 hours and 48 hours treatment of 6-gingerol. For both respective treatment of 6-gingerol, the dose-dependent increase in the expression of cleaved PARP was noted (Fig. 3).

## DISCUSSION

Prostate cancer is common malignancy and one of the common leading causes of cancer-related deaths in men [14,15]. The incidence is also expected to increase in the years to come because prostate cancer occurs usually in men over 50 years of age and the life expectancy is increasing [18]. The unique features of prostate cancer suggest that chemoprevention could be used as a promising approach. For example, the precancerous lesions such as high grade prostatic intraepithelial neoplasia, atypical small acinar proliferation, and proliferative inflammatory atrophy has quite a long period of time (more than 20 years) before progression to cancer [19,20]. The long latency noted in prostate cancer provides a lengthy window of opportunity for intervention by chemopreventive agents [21]. The hope for prostate cancer prevention by natural dietary substances, also known as phytochemicals, has become increasingly popular. Therefore, the action mechanisms of phytochemicals have been recently investigated a lot. The search for new chemopreventive agents which are more effective without toxic effects has generated great interest in identifying phytochemicals for their potential use for prostate cancer [17,20]. 6-Gingerol used in this study is one of the phytochemical agents.

Ginger is one of the most commonly consumed dietary substances in the world and contains a number of pungent ingredients. 6-Gingerol is one of them and possesses various pharmacological activities [4-13]. The present study investigated the effect of 6-gingerol on cell growth and death in LNCaP human prostate cancer cells. We demonstrated that 6-gingerol induced dose- and time-dependent inhibition of cell viability in pancreatic cancer cells. Consistent with the inhibitory effects of 6-gingerol on cellular viability, similar dose- and time-dependent increase in apoptotic cells by 6-gingerol was also observed through flow cytometry analysis. 6-gingerol administered in this study did not exerted cell cycle arrest. The molecular mechanism for 6-Gingerol-induced responses was further presented as the expression of caspase-3 and the degradation of PARP in prostate cancer cells using western blotting. These observations indicated that 6-gingerol may induce apoptosis in LNCaP human prostate cancer cells.

In recent, the apoptosis signaling systems have been known to provide promising targets for the development of novel anticancer agents [22]. Several plant-derived bioactive compounds like phytochemicals have been reported to produce chemopreventive effects through apoptosis in lots of experimental models of carcino-

genesis [11-13,16]. Thus induction of apoptosis is considered as a possible effective mechanism of chemopreventive agents. In the present study, expression of caspase-3 was noted in prostate cancer cells treated with 6-gingerol. The caspases are a family of proteins that plays a central role in the apoptotic process. These belong to a group of enzymes known as cysteine proteases and exist within the cells as inactive proforms [23]. Both the intrinsic and extrinsic pathways have been shown to trigger caspase activation in cells undergoing apoptosis [24]. The active form of caspase-3, a key executioner of apoptosis, is responsible for the cleavage and breakdown of cellular components related to DNA repair and regulation. The PARP, one of the important DNA repair enzymes was identified to be a substrate for the caspases. The ability of PARP to repair DNA damage is inhibited following cleavage of PARP by caspase-3 [25]. Expression of caspase-3 was concomitant with the expression of cleaved PARP by 6-gingerol treatment in this study.

Recently, phytochemicals such as curcumin, resveratrol, and 6-gingerol, which are present in turmeric, red wine and ginger, respectively, have been shown to up-regulate mitogen-activated protein kinase phosphatase-5 in prostate cancer cell lines, implicating potential utility in management of early or advanced prostate cancer [21]. Our results in prostate cancer cells also suggest that 6-gingerol may act as a possible anti-cancer agent. In addition, 6-gingerol caused anti-cancer effects also in another various cancer cell models. For instance, 6-gingerol produced viability reduction in gastric cancer cells [11]. In colorectal cancer cells, cell cycle arrest and caspase-3-dependent apoptosis was induced by 6-gingerol [13]. Taken together, these findings suggest that 6-gingerol could be developed as one of the effective chemopreventive or chemotherapeutic agents in many cancers. Although 6-gingerol induced *in vitro* apoptotic effects in LNCaP prostate cancer cells and many other cancer cell models, more evidences need to be accumulated in order to develop 6-gingerol as a more valid anti-cancer agents for the cancer patients. That is, lots of basic and fundamental biological researches to explore the molecular signaling mechanisms underlying 6-gingerol-induced responses including apoptosis. Studies on the effect of 6-gingerol in animal models are also required. Further clinical trials of chemopreventive action of 6-gingerol need to be performed to provide the ultimate platform for the development of 6-gingerol for therapeutic purposes.

Combinational therapy may act in an additive or synergistic fashion to maximally activate molecular pathways that inhibit carcinogenesis, thereby maximizing cancer prevention while mini-

mizing side effects [26]. In the same manner, combination of a possible chemopreventive agent (such as 6-gingerol) with chemotherapeutic agents may enhance efficacy while reducing toxicity to normal tissues, resulting in better survival. Treatment of human cervical carcinoma cell line, HeLa with ethanolic ginger extract in combination with gemcitabine resulted in significant dose-dependent decrease in cell viability [27]. This suggests the possibility that combinational treatment of 6-gingerol and chemotherapeutic drugs for prostate cancer could reduce toxic side effects on normal cells, while potentiating the efficacy of chemotherapeutic treatment at lower doses. Therefore, further research to test the synergistic effect between 6-gingerol and typical anti-cancer drugs for prostate cancer could be possible.

In conclusion, the present study demonstrated that 6-gingerol induces dose- and time-dependent apoptosis mediated mainly through the expression of caspase-3 and subsequent degradation of PARP in LNCaP human prostate cancer cells. The apoptotic effects induced by 6-gingerol suggests its potential as one of candidate phytochemical agents for chemoprevention or chemotherapy of prostate cancer.

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