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Anti-proliferative effect of ginseng saponins on human prostate cancer cell line

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Abstract

Ginseng is a medicinal herb widely used in Asian countries, and many of its pharmacological actions are attributed to the ginsenosides. In a study of the anti-proliferative activity of ginsenosides using human prostate carcinoma LNCaP cell line, ginsenoside Rg3 displayed growth inhibitory activity. The cells lost its adherent property after incubation in the presence of 250 μ M of ginsenoside for 48h. The expression of biomarker genes, including prostate specific antigen (PSA), androgen receptor (AR) and 5 α -reductase (5 α R), and that of the proliferating cell nuclear antigen (PCNA), were suppressed. Ginsenoside Rg3 induced classic apoptotic morphology and interfered with the expression of apoptosis-related genes, bcl-2 and caspase-3, in LNCaP cells, as demonstrated by fluorescence microscopy, flow cytometry and reverse transcriptase-polymerase chain reaction. Taken our results together, we suggested that ginsenoside Rg3 activated the expression of cyclin-kinase inhibitors, p21 and p27, arrested LNCaP cells at G1 phase, and subsequently inhibited cell growth through a caspase-3-mediated apoptosis mechanism. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: *Panax ginseng* saponins; Prostate cancer cells; Apoptosis; Gene expression; Cell morphology

Introduction

Ginseng has been used for thousands of years in Asian countries, particularly in China, Korea and Japan, for its wide spectrum of medicinal effects such as tonic, immunomodulatory, adaptogenic, and anti-aging activities (1–3). Many of its medicinal effects are attributed to the triterpene glycosides known as ginsenosides. It is noteworthy that the undesirable side effects, if any, are relatively mild and undetectable. There are several ginseng species avail-

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able as medicinal herbs; the best known Asian species is *Panax ginseng* whereas the American species belongs to *Panax quinquefolius*. Both species are widely used in Asian countries for a variety of purposes. It was reported that daily consumption of ginseng extract increased the serum testosterone level and inhibited prostate weight in male rats (4), but no significant expression of PSA was detected after exposing LNCaP cells to ginseng extract (5), and its activity may not be attributed to anti-androgenic activity (6).

Chemical analysis of ginseng revealed the presence of many ingredients, including organic acids, vitamins, sugars, inorganic salts, sterols, oligopeptides, polysaccharides, volatile oils, and saponins. Of these, the saponins (commonly known as ginsenosides) are well studied for their biological properties. In general, the ginseng saponins can be divided into three groups according to the structure of the non-sugar (aglycon) part of the molecule: (a) oleanolic acid type, such as ginsenoside R₀; (b) 20(S)-protopanaxadiol type, such as ginsenosides Ra, Rb, Rc, Rd, Rg3, Rh2 and Rs; and (c) 20(S)-protopanaxatriol type, such as ginsenosides Re, Rf, Rg1, Rg2 and Rh1. To date, more than 30 ginsenosides have been found in the roots and other parts of *P. ginseng*, and a total of over 60 ginsenosides were isolated from members of the *Panax* genus (7). Many of these compounds are responsible for the wide range of medicinal effects of ginseng, such as induction of cancer cell differentiation (8) and angiogenesis (9). On the other hand, cautions have been raised in order to safeguard consumers using these herbal medicines (10–11). As part of a continuing project to evaluate natural products for potential anti-tumor activities, we have studied the effects of seven ginsenosides as well as a saponin fraction of American ginseng using the human prostate cancer LNCaP cell line, with special attention on the molecular mechanism of the cellular effects.

Materials and methods

Chemicals

Seven ginsenosides, including Rd2, Re, Rf, Rg2, Rg3, Rg1, and Ro, were isolated from the root part of *Panax ginseng* in Xu's laboratory at Shenyang Pharmaceutical University (7) and were analytically pure. A sample of quinquenosides, a crude saponin fraction from *P. quinquefolius*, was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

Cell cultures

Human prostate cancer LNCaP cell line and mouse L929 fibroblast cell line were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO₂.

Anti-proliferative activity of ginsenosides

LNCaP cells (1×10^5 cells/0.1 ml/well) were incubated with different concentrations of ginsenosides in 96-well culture plates (Costar, USA) or 8-chamber culture slides (Nunc 177402, USA) for 48h. Cytotoxicity was measured by fluorometric determination of DNA quantity with the fluorochrome Hoechst 33342 (Molecular Probe Inc. H3570, Oregon, USA) using a

fluorometer (Cytofluo 2350, Millipore, USA) (12). Data points represented the mean values and standard deviations of triplicate samples. The LNCaP cells cultured on the chamber slide were stained with acridine orange and FITC-labelled annexin V dyes for morphological and apoptotic studies. The cytotoxicity of ginsenoside Rg3 was further evaluated using L929 fibroblast cells and compared with that of LNCaP cells.

Fluorescent staining for morphological observation

LNCaP cells were treated with different concentrations of ginsenoside Rg3 for 48h, washed briefly with phosphate buffered saline before they were fixed with buffered formalin. The cells were then stained with 0.01% acridine orange in 0.06M phosphate buffer (pH 6.0) and differentiated with 0.1M calcium chloride. Fluorescence micrographs were taken on a fluorescence microscope (Axioskop, Zeiss, Germany) with a 450–490nm excitation block filter and a 520nm barrier filter.

Reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total cellular RNAs of ginsenoside Rg3-treated cells (5×10^6 cells) were isolated using the acid phenol guanidine thiocyanate method (13), purified by phenol-chloroform precipitation before they were reverse-transcribed at 42°C for 50 min using a Superscript™ preamplification system (18089-011, Gibco/BRL, N.Y. USA). Each RT product was subjected to PCR using Thermoprime DNA polymerase (Gibco/BRL, N.Y. USA) and primers for prostate cell markers (prostate specific antigen, androgen receptor and 5 α -reductase), apoptosis-related genes (caspase-3 and *bcl-2*) and cell cycle-related genes (cyclin D1, PCNA, p21 and p27) (Table 1) in a 9700 Perkin-Elmer thermal cycler. The PCR products were separated in a 1.2% agarose gel and the relative intensity against respective β -actin was measured by a densitometer with ImageQuant software (Personal Densitometer, Molecular Dynamics, USA).

Flow cytometry

The ginsenoside Rg3-treated LNCaP cells were fixed with 70% alcohol for 15 min at 4°C before they were stained with 1.0 μ g/ml propidium iodide (PI, Boehringer Mannheim, Germany). The red fluorescence of DNA-bound PI in individual cells was measured at 488nm with an Altra flow cytometer and the results were analyzed using a ExpoII software (Beckman Coulter, USA).

In vitro 5 α -reductase inhibition assay

The *in vitro* 5 α -reductase inhibitory activity of the ginsenosides was measured according to the method of Rhodes *et al.* (22). Briefly, ginsenoside Rg3 (0.1 mM) was incubated with rat prostate homogenate (45 mg/0.1 ml) in the presence of 0.2 μ Ci [1,2,6,7-³H]-testosterone (³H-T, TRK402, Amersham, U.K.), dihydrotestosterone (Fluka 10300, Switzerland), 2.5 μ g propylene glycol (Sigma P3051, St. Louis, USA), 10 μ g NADP (Sigma N0505, St. Louis, USA), 100 μ g glucose 6-phosphate (Sigma G7879, St. Louis, USA) for 1h to enable the 5 α -reduction of ³H-T to ³H-DHT. The product was extracted with chloroform-methanol and separated by thin layer chromatography. The amount of ³H-DHT was measured using a Beckman

Table 1

Primer sequences of prostatic markers, apoptosis-related and cell cycle-related gene

Gene	Primer sequences	Fragment size (bp)	Ref.
Prostate cell marker genes			
PSA	5'-GAGGTCCACACACTGAAGTT-3'	214	14
	5'-CCTCCTGAAGAATCGATTCCCT-3'		
AR	5'-GTGAAAATAGATGGGCTTGA-3'	760	14
	5'-TCACACATGAAGGCTATTGA-3'		
5 α R	5'-CTCCTGGCCATGTTCTC-3'	344	15
	5'-TCAAAAATAGTTGGCTGC-3'		
Apoptosis-related genes			
Caspase-3	5'-ATGGAGAACACTGAAAACCTCA-3'	834	16
	5'-TTAGTGATAAAAATAGAGTTC-3'		
Bcl-2	5'-CAGCTGCACCTGACG-3'	241	17
	5'-ATGCACCTACCCAGC-3'		
Cell cycle-related genes			
Cyclin D1	5'-TGGATGCTGGAGGTCTGCCAGGAA-3'	573	18
	5'-GGCTTCGATCTGCTCCTGGCAGGC-3'		
PCNA	5'-GCTGACATGGGACACTTA-3'	160	19
	5'-CTCAGGTACAACTTGGTG-3'		
p21	5'-CTTTGACTTCGTCACGGAGAC-3'	253	20
	5'-AGGCAGCGTATATCAGGAGAC-3'		
p27	5'-AAGCACTGCCGGGATATGGA-3'	297	20
	5'-AACCCAGCCTGATTGTCTGAC-3'		
β -actin	5'-CCTTCTACAATGAGC-3'	594	17
	5'-ACGTCACACTTCATG-3'		

scintillation counter. All samples were performed in triplicates and expressed in percentage of inhibition by normal prostate tissues.

Results

Cytotoxicity of ginsenosides

Of the eight ginsenoside samples tested, only Rg3 displayed a dose-dependent anti-proliferative activity on LNCaP cells ($IC_{50} = 650 \mu\text{M}$) (Fig. 1). Untreated LNCaP cells appeared as spindle shape, attached smoothly on the culture surface, and some of the cells grouped together to form colonies (Fig. 2a). Following treatment with ginsenoside Rg3 for 48h, the cells changed to round shape and made fewer cellular contacts (Fig. 2b); they lost their surface morphology and died at a ginsenoside Rg3 concentration of 500 μM (Fig. 2c). Immunofluorescence analysis demonstrated that cell death is mediated through apoptotic changes of the LNCaP cells (Fig. 2d). Since cytotoxicity was undetected in other ginsenoside samples ($IC_{50} > 2 \text{ mM}$) (data not shown), only Rg3 was subjected to further cytotoxic assays. However, no anti-proliferative activity was observed in L929 fibroblast cells, in which only about 18% growth inhibition was observed at 500 μM of ginsenoside Rg3.

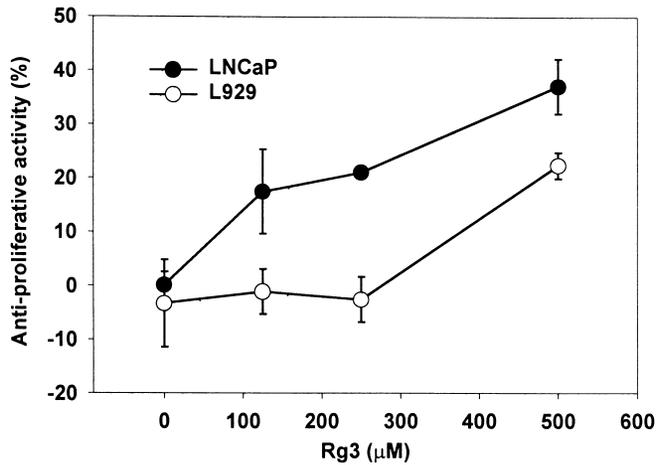


Fig. 1. The anti-proliferative activity of ginsenoside Rg3 on prostate cancer LNCaP cells. Cells were cultured with different concentrations of ginsenoside Rg3 for 48h and the cell number was estimated by determination of fluorometric DNA quantitation using the fluorochrome Hoechst 33342. Each data point represents the mean and standard deviation of triplicates.

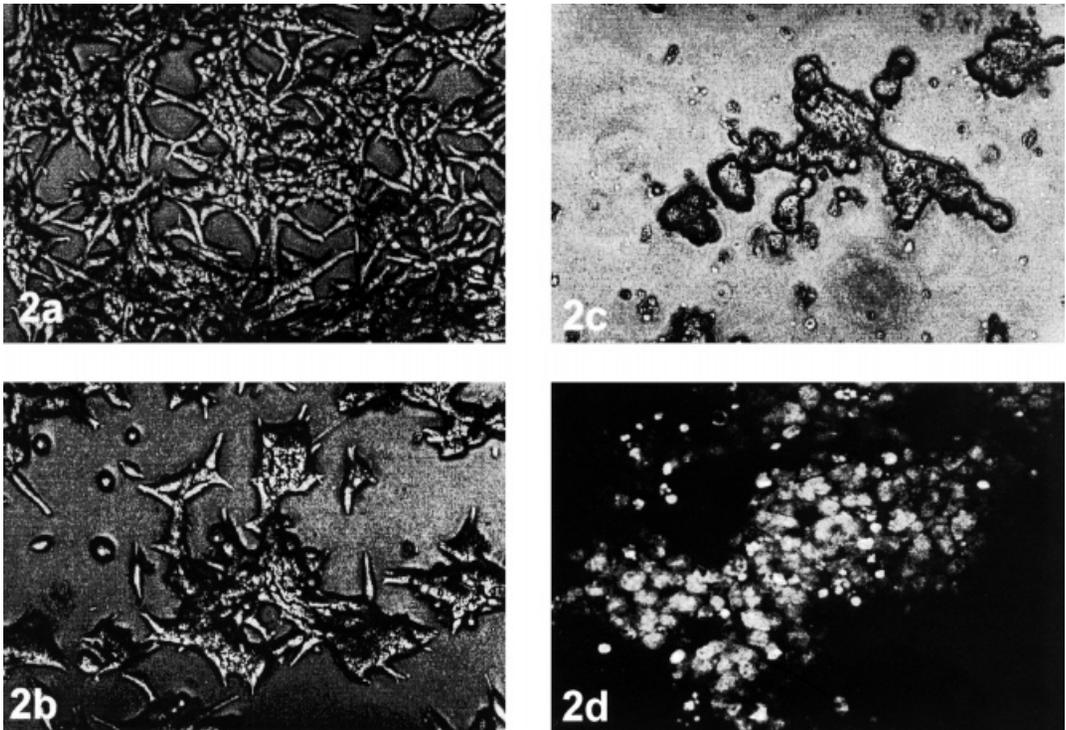


Fig. 2. The morphological changes of human prostate cancer LNCaP cells treated with ginsenoside Rg3 for 48h. Normal LNCaP cells proliferated and formed colonies (a), but some of them lost their adherent activity in the presence of 250 μM ginsenoside Rg3 (b). The cells lost their surface morphology, showed apoptotic changes (arrows) and subsequently died at 500 μM of ginsenoside Rg3 (c-d). (400 \times)

Effect of ginsenoside Rg3 on cellular marker expression of LNCaP cells

Prostate specific antigen (PSA) is a prostate differentiation marker which is up-regulated by binding androgens to the androgen receptors (AR). The 5 α -reductase (5 α -R) converts testosterone into the more potent dihydrotestosterone which binds to androgen receptor for actions. Thus, the expression of these three markers represents the activity of the androgen-dependent prostate cancer LNCaP cell line. Our results (Fig. 3) clearly show that ginsenoside Rg3 exhibited a dose-dependent inhibitory activity on the expression of all three marker genes.

Effect of ginsenoside Rg3 on the cell cycle of LNCaP cells

LNCaP cells were exposed to increasing concentrations of ginsenoside Rg3 (125, 250 and 500 μ M) for 48h, and the growth of the cells was analyzed using flow cytometry (Fig. 4). In the absence of ginsenoside Rg3, the cell populations at G1, S and G2/M phases were found to be 65%, 11.5% and 21.3%, respectively. Following an exposure to 125 μ M of ginsenoside Rg3, the proportions were reduced to 4.2% and 11.2% for the S and G2/M phases (Fig. 5), accompanied by a concomitant increase of the G1 phase (75.2%). This observation led to a suggestion of G1 arrest or slowdown. DNA fragmentation (12% and 30% for 250 and 500 μ M, respectively) was observed when the cells were exposed to ginsenoside Rg3 at 250 μ M or above. The expression of proliferating cell nuclear antigen and cyclin-kinase D1 in LNCaP cells diminished in the presence of 125 μ M ginsenoside Rg3; in comparison, the transcription of cyclin-kinase inhibitors p21 and p27 was increased constitutively at 250 and 500 μ M concentrations of the ginsenoside (Fig. 3).

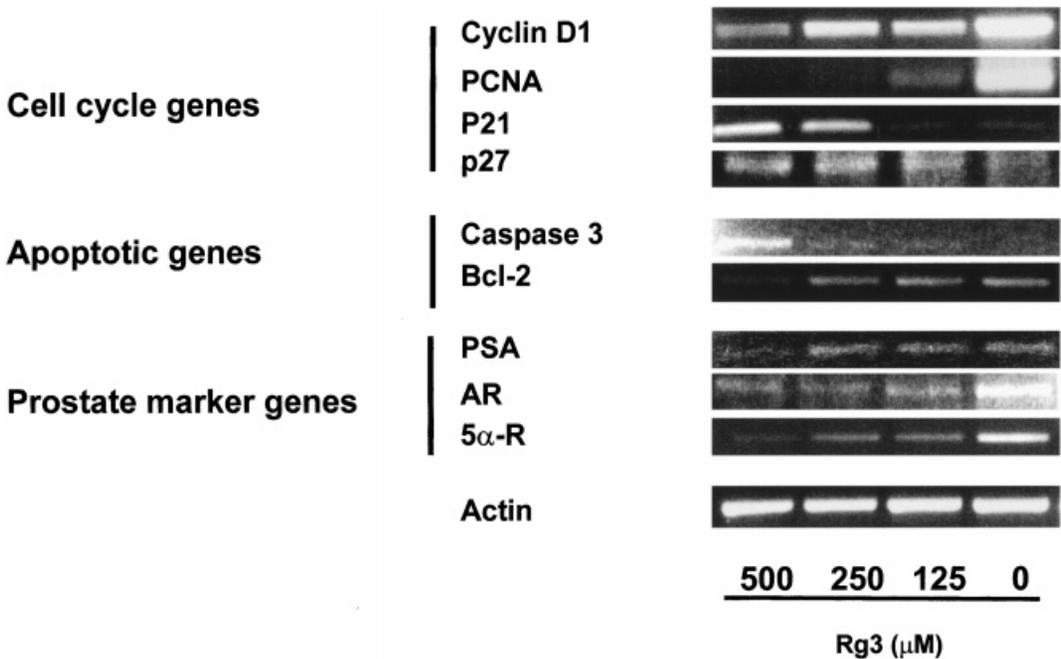


Fig. 3. RT-PCR analysis of the expression of cell cycle genes, apoptotic genes and prostate marker genes in LNCaP cells treated with ginsenoside Rg3 for 48h.

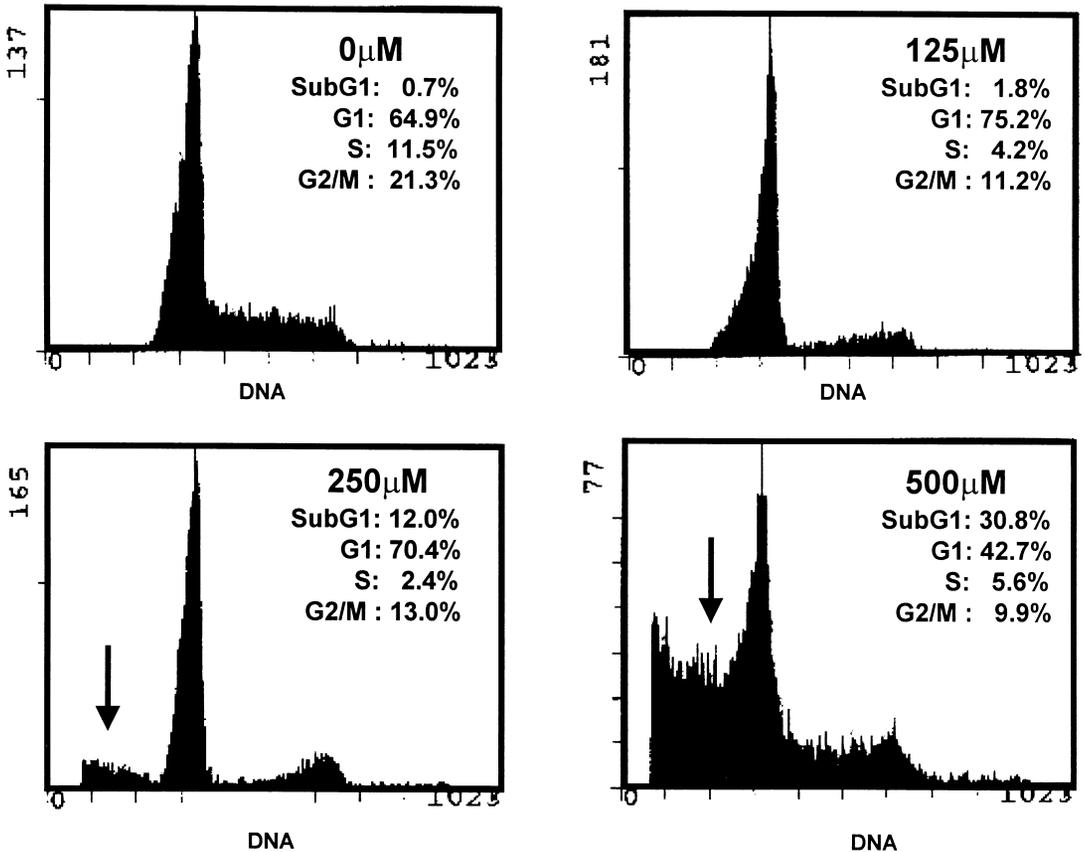


Fig. 4. DNA content frequency histograms of LNCaP cells treated with ginsenoside Rg3 for 48h. A normal DNA pattern was observed in cells prior to ginsenoside treatment, but the level of subG1 (arrows) was increased in cells after treatment with ginsenoside Rg3 at 250 μ M and 500 μ M.

In vitro 5 α -reductase inhibition assay

Of the 8 ginsenoside samples tested, ginsenoside Rg 3 possessed the most potent *in vitro* inhibitory activity on 5 α reductase obtained from rat prostate homogenate (data not shown). Fig. 6 shows a dose-dependent decrease of 5 α reduction of testosterone into ³H-DHT, indicating that ginsenoside Rg3 can exert a direct inhibition on the enzyme.

Discussion

Prostatic hypertrophy and prostate cancer are the most prevalent proliferative disorders in aging male reproductive system. The enlargement of the prostate compresses the urethra, prevents the bladder from emptying and causes the subsequent micturition problem. Since androgen plays a critical role in regulating the growth and differentiation of prostate cells, hormone therapy becomes the standard treatment for primary prostate cancer. However, re-

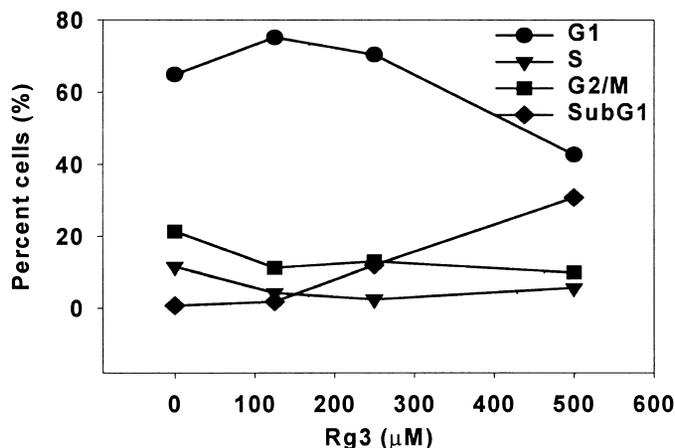


Fig. 5. The cell cycle distribution of LNCaP cells treated with ginsenoside Rg3 for 48h. The cell population in G1 phase was decreased with a concomitant increase of subG1, indicating apoptotic changes in cells exposed to increasing doses of Rg3 (250 and 500 μM).

current tumor normally develops in years when the patients no longer respond to hormone therapy. Chemotherapy with cytotoxic agents has thus been suggested as an alternative growth inhibitor of hormone-independent prostate cell (23). However, due to the slow proliferation of the prostate cancer cells, the effectiveness of cytotoxic agents towards these cells is greatly diminished (24). Agents capable of inducing apoptosis, inhibiting cell proliferation, or modulating signal transduction are currently used for the treatment of cancer (24), and a combination use of multiple chemopreventive agents or agents with multiple targets are considered to be more effective (25). Herbal therapy has thus been introduced partly because herbs consist of constituents with multiple targets, and partly because there is a long tradition

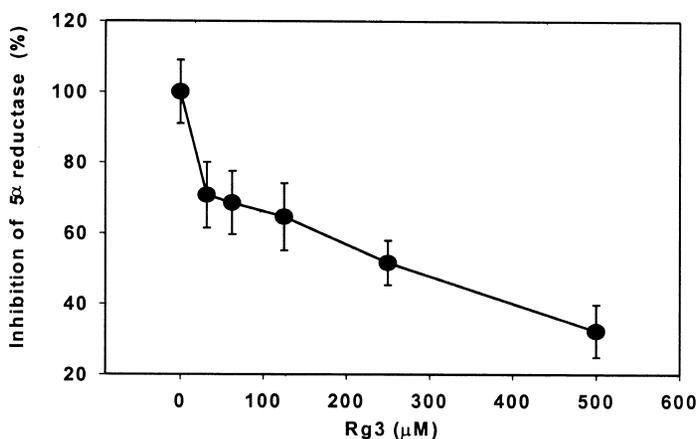


Fig. 6. A dose-dependent *in vitro* inhibition of 5 α reductase activity by ginsenoside Rg3. Each data point represents the mean and standard deviation of triplicates.

of using herbs in Asian and European countries (26). Ginseng is one of the commonly used herbal medicines the underlying mechanism of which is not clear (27). Public concerns over safe herbal uses have been raised (11,28). Recent investigations have shown that ginseng extract and its components could suppress tumor promoting activity (29), induce apoptosis in cancer cells (30–31), interfere with cell cycle progression (32), enhance immune activity (33) and suppress tumor angiogenesis (34). The present study has further demonstrated that out of 8 selected ginseng saponins, ginsenoside Rg-3 was the most potent growth inhibitor against LNCaP cells. It inhibited cell proliferation by suppressing the cell cycle progression genes, e.g. PCNA and cyclin kinase D1, associated with an increased expression of cyclin kinase inhibitor genes, e.g. p21 and p27, leading to a G1 arrest (at 125 μ M of Rg3) and apoptosis at higher dose (250 μ M). The cell death was apparently executed by down-regulating *Bcl-2* gene and concomitantly activating caspase-3 gene as demonstrated by RT-PCR analysis of apoptosis-related gene expression.

The LNCaP cells contain androgen receptors and their growth is androgen dependent. Since the steroid action on target cells is mediated by intracellular receptor protein, it has been inferred that loss of steroid receptors will cause a loss of the corresponding steroid response or vice versa. Active LNCaP cell proliferation involves androgen receptor and 5 α reduction of testosterone (7) with a concomitant secretion of prostate specific antigen (PSA). Two isoforms of 5 α reductases, namely type 1 and type 2, have been sequenced in human prostate; type 1 is expressed in stroma and epithelium while type 2 is only found in stroma (35). The 5 α reductases convert testosterone into potent dihydrotestosterone. Our results demonstrated that ginsenoside Rg3 can down-regulate the expression of all these three marker genes in LNCaP cells, indicating that the compound not only interferes with the cell cycle progression but also affects the conversion of testosterone into more potent dihydrotestosterone. A cell-free *in vitro* 5 α reductase inhibition assay was performed in an effort to delineate that the reduced expression of 5 α reductase gene is a consequence of cytotoxic effect of ginsenoside Rg3 on LNCaP cells. Our preliminary data has demonstrated a direct inhibitory effect on 5 α reductase. Since the androgenic induction of PCNA is mediated through the androgen receptor (36) and PCNA is an auxiliary protein for DNA polymerase, a decrease of LNCaP proliferation is resulted as a consequence of the suppression of 5 α reduction induced by the ginsenoside. Rg3 is a minor component, only 3–4% of total ginsenosides, in ginseng radix (37), which not only inhibited *in vitro* tumor cell growth in the present study but also tumor metastasis in mice bearing B16-BL6 melanoma and colon 26-M3.1 carcinoma (34,38). Taken all these data into account, we believe that ginsenoside Rg3 exerts multiple anti-proliferative actions towards the androgen-dependent LNCaP cells by inhibiting the 5 α reduction of testosterone, preventing cell progression, and inducing a caspase-3 mediated apoptosis.

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