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# Effect of petroleum ether extract of *Panax ginseng* roots on proliferation and cell cycle progression of human renal cell carcinoma cells

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Abbreviation: RCC, renal cell carcinoma; GX-PE, petroleum ether extract of Korean Ginseng

## Abstract

***Panax ginseng* roots have long been used as a medi-cinal herb in oriental countries. We have investigated anti-proliferative effects of lipid soluble *Panax ginseng* components on human renal cancer cell lines. Petroleum ether extract of *Panax ginseng* roots (GX-PE) or its partially purified preparation (7:3 GX) was added to cultures of three human renal cell carcinoma (RCC) cell lines, A498, Caki-1, and CURC II. Proliferation of RCC cells was estimated by a [$^3$H]thymidine incorporation assay and cell cycle distribution was analyzed by flow cytometry. GX-PE, 7:3 GX, panaxydol and panaxynol inhibited prolife-ration of all three RCC cell lines in a dose dependent manner *in vitro* with an order of potency, 7:3 GX> panaxydol> panaxynol = GX-PE. Additive effect of interleukin 4 was also demonstrated, most prominently in Caki-1 which responded poorly to GX-PE alone. Analysis of cell cycle in CURC II and Caki-1 treated with GX-PE demonstrated increase in $G_1$ phase population and corresponding decrease in S phase population. The present study demonstrated that proliferation of human RCC cell lines were inhibited by lipid soluble components of *Panax ginseng* roots by blocking cell cycle progression at $G_1$ to S phase transition.**

**Keywords:** *Panax ginseng*, renal cell carcinoma, cell cycle, panaxynol, panaxydol, interleukin 4

## Introduction

Roots of *Panax ginseng* (*Panax ginseng* C. A. Meyer; Korean ginseng) have been used as a medicinal herb for more than 2,000 years in oriental countries including China, Korea, and Japan. Although pharmacological activities of *Panax ginseng* have been known for sometimes, ranging over a wide spectrum of effectiveness such as anti-fatigue and anti-stress effects and promotion of longevity, the scientific bases for its understanding is still premature. In recent years, however, active scientific research has been promoted to understand the chemical composition, pharmacological and biological activities of *Panax ginseng* (Hwang, 1993; Kim *et al*., 1993; Stancheva *et al*., 1993; Kimura *et al*., 1994; Nah *et al*., 1995).

The most extensively studied component of *Panax ginseng* for its activity is a glycoside, saponin (Brekman and Dardymov, 1969). However, others such as phenolic acids, alkaloids and lignans may also be responsible for many of the important effects of *Panax ginseng*. Petroleum ether extracts of *Panax ginseng* have been reported to possess anti-proliferative effects on various cancer cell lines including murine sarcoma, murine leukemia, human colon carcinoma, and human ileocecal adenocarcinoma cell lines (Hwang and Cha, 1978; Lee and Hwang, 1986). Active cytotoxic compounds in the petroleum ether extract in some of these cell lines have been shown to be polyacetylenic compounds such as panaxynol, panaxydol, and panaxytriol (Matsunaga *et al*., 1990).

Renal cell carcinomas (RCC) are refractory to most of chemotherapeutic drugs and at present, none of the therapeutic modalities are effective in metastatic RCC which occur in one third of RCC patients at the time of diagnosis. Optional immunotherapy with interferons or interleukin 2 also results in tumor regression only in 10-15% of RCC patients. Therefore, in an effort to develop a new therapeutic agent for RCC, we have tested whether lipid soluble components of *Panax ginseng* inhibit growth of RCC cell lines *in vitro* and investigated the mechanism of anti-proliferative effects by analyzing cell distribution at different stages of a cell cycle. In addition, combination of lipid soluble components of *Panax ginseng* and interleukin 4 as a possible anticancer drug for metastatic RCC has been also investigated.

## Materials and Methods

### Materials

Steamed and dried roots of *Panax ginseng*, or Red

Ginseng, panaxynol and panaxydol were provided by the Korea Ginseng and Tobacco Research Institute (Taejon, Korea). Recombinant human interleukin 4 was purchased from BioSource International Inc. (Camarillo, CA, USA).

### Preparation of GX-PE and 7:3 GX

Powdered Red Ginseng was extracted with petroleum ether by shaking vigorously at 40˚C. The extract was concentrated with a vacuum evaporator and the residual petroleum ether was removed by flowing $N_2$ gas over the sample. Seven to three (7:3) GX was prepared by a method described by Lee *et al*. (1986). Before adding to the culture, GX-PE and 7:3 GX was dissolved in ethyl alcohol at 166 mg/ml and further diluted in the culture medium.

### Cell lines and cell culture

Caki-1, A498, and CURC II are human RCC cell lines. Caki-1 and A498 were obtained from the Korean Cell Line Bank (Seoul, Korea). CURC II was obtained from Woo Chul Moon (Chungang University, Seoul, Korea). MRC-5 is a nontransformed human lung fibroblast cell line. All cell lines except MRC-5 were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. MRC-5 was cultured in MEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin.

### Primary culture of synovial cells

Synovial tissue of rheumatoid arthritis patient was washed in PBS (phosphate buffered saline) and cut into small pieces. Jolik's MEM containing 1-1.5mg/ml dispase II (grade II, Boehringer Mannheim, Indianapolis, IN, USA) was added and incubated at 37˚C for 30 minutes with constant agitation. Medium containing dissociated synovial cells was collected and placed into a tube containing an equal volume of RPMI 1640 with 15% fetal bovine serum. Dispase treatment was repeated four times. Synovial cells were centrifuged on the Ficoll-hypaque solution, washed and cultured in RPMI 1640 containing 15% fetal bovine serum and anibiotics.

### Silica gel thin layer chromatography (TLC)

Silica gel TLC was performed as described by Hwang *et al*. (1978). Briefly TLC plates (Polygram sil G/UV254, Brinkman, NY, USA) were heated for 2 h at 120˚C and 25-100 μg each of samples were applied. After developing in a petroleum ether/ethyl ether/acetic acid (90:10:1, v/v) solvent, spots were located by spraying 30% $H_2SO_4$, 5% ethanol and drying at 120˚C for 5 min.

### Proliferation assay

[$^3$H]thymidine incorporation assay was used to analyze proliferation of RCC cell lines. Cells were plated in a 96 well plate at a density of 1-5 $\times 10^3$ cells/well and various preparations of lipid soluble ginseng extract were added at different concentrations. Cells were cultured for 72 h and 1 μCi/well of [$^3$H]thymidine (Amersham, Arlington Heights, IL, USA) was added. After further incubating for 18 h, cells were harvested onto a glass fiber filter and [$^3$H]thymidine incorporation was measured with a β-scintillation counter (Phamacia, Uppsala, Sweden). Percent inhibition of tumor cell growth was calculated as follows:

$$\% \text{ inhibition} = [1 - \frac{\text{CPM of treated cells}}{\text{CPM of control cells}}] \times 100$$

### Cell cycle analysis

The DNA content of cell suspensions prepared by trypsin treatment was determined by propidium iodide labeling with the use of a cell cycle analysis kit (Beckton Dickinson, Mountain View, CA, USA) following the manufacturer's instructions. Finally, propidium iodide was added to the cell suspension and incubated in the dark for 10 min. Total DNA content per cell was assessed by analysis of fluorescence at 585 nm by using FACScan flow cytometer, equipped with pulse width area doublet discriminator (Beckton Dickinson). A minimum of 1 $\times 10^4$ cells/sample were analyzed. The resulting histogram was analyzed by using the Cellfit analysis program (Beckton Dickinson).

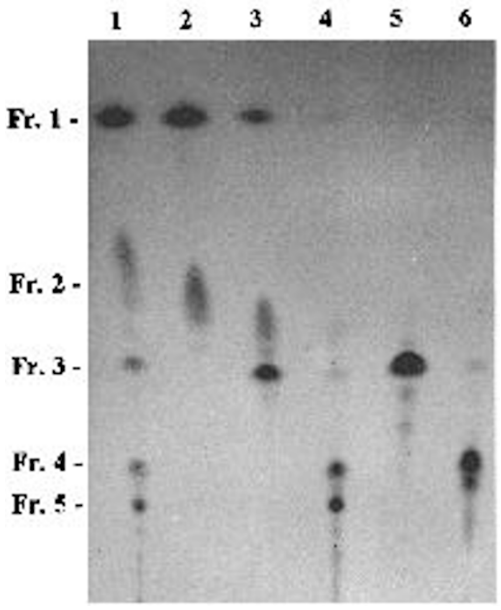



**Figure 1.** Silica gel TLC of GX-PE and 7:3 GX. TLC was performed as described in materials and methods. Lane 1, GX-PE; lane 2, 9:1 GX (components of GX-PE eluted from silicic acid column chromatography with 9:1 petroleum ether:ethyl ether mixture); lane 3, 8:2 GX; lane 4, 7:3 GX; lane 5, panaxynol; lane 6, panaxydol.

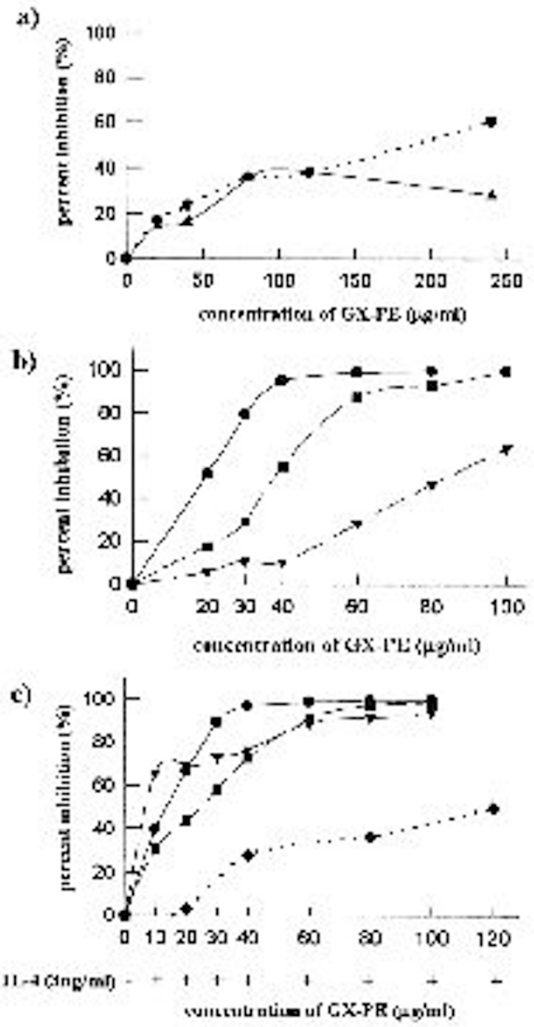
## Results

### TLC analysis of lipid soluble components of *Panax ginseng*

On a silica gel TLC, GX-PE was separated into 5 fractions (designated as fractions 1 to 5 according to the order of $R_f$ value) (Figure 1). Seven to three (7:3) GX contained fractions 4 and 5 of GX-PE. Nine to one (9:1) GX resulted in fractions 1 and 2; 8:2 GX resulted in fraction 3 as well as traces of fractions 1 and 2. Panaxynol and panaxydol corresponded to fractions 3 and 4 of GX-PE, respectively.

### Effects of GX-PE on proliferation of RCC cell lines

GX-PE inhibited growth of A498, Caki-1, and CURC II in a dose dependent manner (Figure 2b). A498 was most sensitive to growth inhibitory effects of GX-PE. At a concentration of 40 μg/ml, growth of A498 was inhibited by 95% and CURC II, by 55%. Caki-1 responded poorly and proliferation was inhibited by 10% at the same concentration. The extent of growth inhibition of synovial cells and MRC-5 was comparable to that of Caki-1 up to 80 μg/ml GX-PE (Figure 2a, b). However, at higher concentrations, growth inhibition by GX-PE in these cell lines did not increase as much as in Caki-1. In contrast, GX-PE completely inhibited growth of A498 and CURC II at 80 μg/ml and the growth of Caki-1 was inhibited by over 60% at 100 μg/ml. These results show that lipid soluble components of *Panax ginseng* roots inhibit proliferation of human RCC cell lines directly and growth of transformed cells is preferentially inhibited especially at a high concentration of GX-PE.




### Additive effect of GX-PE and interleukin 4 on growth inhibition of RCC cell lines

In a previous study, we reported that recombinant human interleukin 4 has direct anti-proliferative effects on human RCC cell lines (Cheon *et al*., 1996). Since Caki-1 was relatively resistant to anti-proliferative effects of GX-PE but sensitive to interleukin 4, we tested whether interleukin 4 could be used to increase and/or complement growth inhibitory effects of GX-PE. Indeed, there was an additive effect between IL-4 and GX-PE (Figure 2c). When 3 ng/ml of interleukin 4 was added to cultures containing suboptimal concentrations (10, 20, and 30 μg/ml) of GX-PE, proliferation of RCC cell lines decreased by additional 60 to 70% in Caki-1 and 10 to 30% in CURC II and A498. Thus, an additive effect of interleukin 4 was found in all RCC cell lines but it was the most prominent in Caki-1. In contrast, interleukin 4 did not show any additive effect in inhibiting the growth of MRC-5. This suggests that GX-PE and interleukin 4 may be used together to increase a response rate in RCC treatment.

### Effects of 7:3 GX on proliferation of RCC cell lines

GX-PE was purified for its anti-proliferative effects on cancer cells by silicic acid column chromatography. The fraction eluted by 7:3 petroleum ether:ethyl ether was highly effective in inhibiting growth of RCC cell lines (Figure 3a) and called 7:3 GX. At a 10 μg/ml concentration of 7:3 GX, the proliferation of A498 and CURC II was decreased by 98% compared to less than 10% inhibition with GX-PE at the same concentration. In Caki-1, 51.7% of growth inhibition was observed at 10 mg/ml 7:3 GX compared

**Figure 2.** Effects of GX-PE on proliferation of human RCC cell lines. GX-PE was added to the culture medium and proliferation was measured by [$^3$H]thymidine incorporation. **a)** synovial cells (triangles), MRC5 (circles); **b)** A498 (circles), Caki-1 (triangles) and CURC II (squares). **c)** Varying concentrations of GX-PE and 3 ng/ml of human interleukin 4 was added to cultures of A498 (circles), Caki-1 (triangles), CURC II (squares), and MRC5 (diamonds).

to 2.6% with GX-PE. Overall, 7:3 GX is 5 to 15 fold more effective in inhibiting proliferation of human RCC cells and seems to contain most, if not all, of the anti-proliferative activity of GX-PE.

### Effects of panaxydol and panaxynol on proliferation of human RCC

Panaxydol and panaxynol, the polyacetylenic compounds, are components of GX-PE. Panaxydol (fraction 4 of GX-PE on TLC; Figure 1) has 2-6 fold stronger effects on inhibiting proliferation depending on RCC cell lines (Figure 3b). However, growth inhibitory effects of panaxydol was the most prominent in CURC II whereas that of GX-PE was more potent in A498. At 10 mg/ml of panaxydol, proliferation of A498 and CURC II was inhibited by 43.5% and 80.5%, respectively. Although panaxynol (fraction 3 of GX-PE on TLC; Figure 1) was not included in 7:3 GX, it also inhibited growth of all the RCC cell lines, albeit in a much lesser extent than panaxydol or 7:3 GX (Figure 3c).

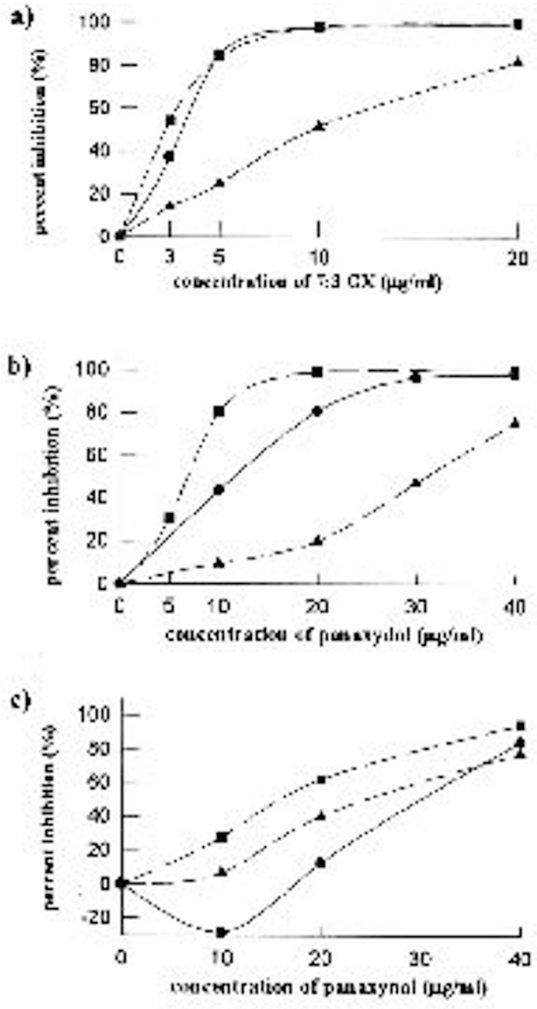



**Figure 3.** Effects of 7:3 GX, panaxydol and panaxynol on proliferation of A498 (circles), CURC II (squares), and Caki-1 (triangles).

### Effects of GX-PE on a cell cycle

To assess effects of GX-PE on cell cycle, CURC II and Caki-1 were treated with GX-PE for 72 h and analyzed for DNA distribution by flow cytometry. Both CURC II and Caki-1 incubated with GX-PE had reduced S-phase and increased $G_0/G_1$ phase populations compared to control cells incubated in culture medium only. As seen in Table 1, the effect of GX-PE on cell cycle was dose dependent. This suggests that GX-PE inhibits growth of RCC cells by blocking cell cycle progression at $G_1$-S transition.

## Discussion

Here we demonstrated that lipid-soluble components from *Panax ginseng* roots inhibit proliferation of human RCC cell lines *in vitro* and that the growth inhibition accompanies a block in cell cycle progression at G1 phase. Anti-proliferative activity was tested with GX-PE, 7:3 GX, panaxydol and panaxynol. Seven to three (7:3) GX and panaxydol showed stronger growth inhibitory effects than GX-PE. Most of the anti-proliferative activity of GX-PE seem to be retained in 7:3 GX since 7:3 GX was 5- to 15-fold more effective than GX-PE. However, panaxynol which is not included in 7:3 GX (Figure 1), also exhibited some

**Table 1.** Cell cycle distribution of Caki-1 and CURC II treated with varying concentrations of GX-PE for 72 h.

| GX-PE (μg/ml) | Cell cycle distribution (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Caki-1 | | | | CURC II | | | |
| | $G_0/G_1$ | S | $G_2$+M | $G_0/G_1$: S ratio | $G_0/G_1$ | S | $G_2$+M | $G_0/G_1$: S ratio |
| 0 | 48.1 | 31.0 | 20.9 | 2.30 | 46.9 | 28.9 | 24.1 | 1.62 |
| 40 | - | - | - | - | 51.5 | 23.3 | 25.3 | 2.21 |
| 80 | 62.1 | 15.1 | 22.8 | 4.11 | 54.1 | 15.8 | 30.0 | 3.42 |

anti-proliferative activity. Fraction 2 of GX-PE, obtained by preparative silica gel TLC, did not inhibit growth of RCC cells (data not shown). Growth inhibitory effects of panaxydol (fraction 4 of GX-PE on TLC) was stronger than GX-PE but weaker than 7:3 GX. Since 7:3 GX contains fractions 4 and 5, this suggests that fraction 5 of GX-PE on TLC also has a strong anti-proliferative effect.

The efficiency of inhibiting proliferation of different RCC cell lines may vary with different components of GX-PE. Anti-proliferative effect of GX-PE was greatest on A498 whereas that of panaxydol and panaxynol was greatest on CURC II. Since 7:3 GX inhibits growth of A498 and CURC II at a similar degree, it will be of interest to see if TLC fraction 5, one of the two fractions of 7:3 GX, is most potent on A498. This also suggests a possibility that different components of GX-PE inhibit growth of cancer cell lines by different mechanisms.

Previously we and others reported that recombinant human interleukin 4 inhibited proliferation of human renal cancer cell lines (Hoon *et al*., 1991; Cheon *et al*., 1996). Here we showed that interleukin 4 and GX-PE have an additive effect in inhibiting growth of human RCC but not in a nontransformed human lung fibroblst cell line, MRC-5. This suggests that interleukin 4 and GX-PE inhibit growth of RCC cells through different pathways. This notion is further supported by the finding that anti-proliferative effects of GX-PE is greatest in A498 whereas that of interleukin 4 is greatest in Caki-1. Therefore, GX-PE and interleukin 4 can be used together to increase and/ or complement effectiveness of RCC treatment and to reduce side effects by being able to decrease a dose of each agent.

To investigate whether GX-PE preferentially inhibit growth of transformed cells, rheumatoid synovial cells were used as a nontransformed control cells. The result suggests that, especially at a high concentration (over 100 μg/ml), GX-PE preferentially inhibit growth of transformed cells. Although toxicity of GX-PE and/or 7:3 GX needs to be investigated further, the results presented in this paper suggest that GX-PE and 7:3 GX may make promising drugs for metastatic RCC.

## Acknowlegements

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