

## Research Article

### Ginseng Extract Enhances Anti-cancer Effect of Cytarabine on Human Acute Leukemia Cells

Yiju Hou, Xiaodong Liu, Zhonghai Yuan and Yan Li

Department of Laboratory Medicine, Jilin Medical College, Jilin 132013, China

**Abstract:** Ginseng as a traditional medicine is well known to exhibit various pharmacological effects. Ginsenoside Rg3 is the active ingredient extracted from ginseng. The pharmacological modulatory effects of Rg3 on multidrug resistant cancer cells are reported in the present study. Cytarabine is a chemotherapeutic agent for the treatment of acute leukemia. However, this compound has serious side effects at high doses, for example hematopoiesis depression. In this study, using hl60 human leukemia cells, we investigated the possible synergistic anti-cancer effects between ginseng extract Rg3 and cytarabine on acute myeloid leukemia cells. Results of this study demonstrate that Rg3 can enhance the anti-proliferation effect of cytarabine on hl60 cells and may decrease the dosage of cytarabine needed for acute myeloid leukemia treatment.

**Keywords:** Anti-cancer, cytarabine, ginseng, leukemia

#### INTRODUCTION

Dietary supplementation has been prevalent in cancer therapy for a long time. Ginsenoside Rg3 is a chemical component extracted from *Panax ginseng* and exhibits a number of biological activities, including anti-oxidant, anti-cancer, anti-stress and anti-diabetes activities (Lü *et al.*, 2009). Acute leukemia is a hematological malignancies and its incidence has been increasing. Chemotherapy is the most important treatment for the patients with cancer. However, unlike most types of medications, chemotherapy drugs often cause serious or intolerable side effects. In fact, the side effects of chemotherapy often limit the efficiency of the drugs (Zhang *et al.*, 2006). We have investigated the effects of botanical extracts on reducing chemotherapeutic side effects and found that *Panax ginseng* extract can attenuate cisplatin-induced nausea and vomiting. Additionally, the extract from *Panax ginseng* enhanced the anti-proliferation effect of cisplatin on human breast cancer cells (Nahleh and Tabbara, 2003), suggesting that it possesses its own anti-cancer effect. Cytarabine is a chemotherapy drug that interferes with the growth of cancer cells. It is the commonly used drug in the treatment of human acute myeloid leukemia. However, this drug has serious side effects including nausea, fatigue and a decrease in the number of blood cells. Studies have found that for the treatment of relapsed acute myeloid leukemia, higher doses of cytarabine are no more effective yet increase side effects. Therefore, to find the chemotherapy sensitizer is a very meaningful thing (Lee *et al.*, 2009).

To investigate whether Rg3 can inhibit cancer cell growth, we examined the susceptibility of human leukemia cells hl60 to treatment with Rg3 and Rg3-induced apoptosis. Rg3 dose-dependently inhibited cancer cell growth through induction of apoptosis. In a further study of compounds in leukemia cells, we used the minimum effective dose, values in combined treatments of Rg3 with conventional agent's cytarabine (Abdoulaye *et al.*, 2013). Compared to treatment with Rg3 or chemotherapy alone, combined treatment was more effective in the inhibition of cancer cell growth and induction of apoptosis. The expression of apoptotic cell death proteins Bax was significantly enhanced, but the expression of anti-apoptotic genes Bcl-2 was significantly inhibited by the combined treatment compared to Rg3 or cytarabine alone. These results indicate that ginsenoside Rg3 enhances anti-cancer effect of cytarabine on human acute leukemia cells. Thus, ginsenoside Rg3 could be useful as an anti-cancer or adjuvant anti-cancer agent.

#### MATERIALS AND METHODS

**Materials:** Ginsenoside Rg3 (molecular weight: 785.01), a major ginsenoside extracted from *Panax ginseng*, was obtained from a Hongjiu bio-tech company in Jilin province and its structure is shown in Fig. 1A. Cytarabine (molecular weight: 243.21) was Shanghai Hualian pharmaceutical products and its structure is shown in Fig. 1B. Dimethyl Sulfoxide (DMSO) and MTT were produced by Sigma Corporation. Calf serum was Hangzhou sijiqing company's products, RPMI-1640 media purchased from

**Corresponding Author:** Yan Li, Department of Laboratory Medicine, Jilin Medical College, Jilin 132013, China

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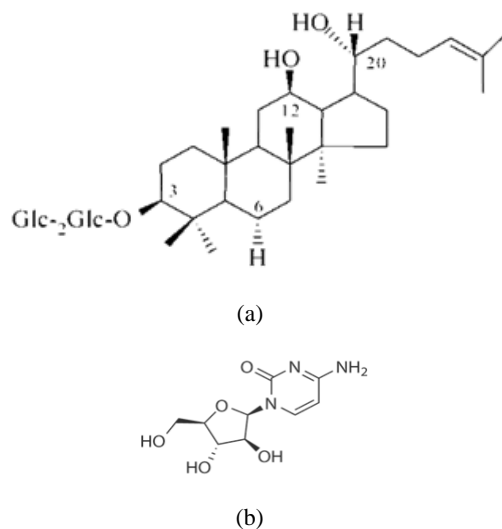


Fig. 1: Structures of Rg3 (a) and Cytarabine (b)

United States Gibco Corporation. Ginsenoside Rg3 was dissolved in 0.01% DMSO and used for treatment.

**Cell culture and treatment groups:** Human acute myeloid leukemia cells were provided by the Cell Bank of Shanghai Institute of Cell Biology. The hl60 cells were cultured with RPMI1640 medium containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 10% FBS and 50 IU penicillin/streptomycin, in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Hl60 cells growth in liquid suspension culture. Hl60 leukemia cells were routinely cultured and passaged. When grown to logarithmic phase, hl60 cells were divided into three groups according to the way of treatment: Rg3 (2.0, 4.0, 8.0, 16.0 µg/mL), cytarabine (5, 10, 20, 40 µg/mL), Rg3 combined with cytarabine (4.0 µg/mL Rg3+ 5, 10, 20, 40 µg/mL cytarabine).

**Cell proliferation analysis:** Ginseng extract was dissolved in Dimethyl sulfoxide and cytarabine was diluted with water for injection. Rg3 and cytarabine stock solutions were stored at 4°C before use. Hl60 leukemia cells were prepared into suspension and plated in 96-well plates (3×10<sup>5</sup> cells/well) with 100 µL each wells during the logarithmic phase. After 6 h, various concentrations of Rg3 and cytarabine were added to the wells. Controls were exposed to culture medium containing the same quantity of DMSO without drugs. The total volume added to each well should be 200 µL. After treatment with Rg3 and/or cytarabine for 48h, cell growth was evaluated using an MTT assay, which is based on the conversion of MTT to form crystals by mitochondrial dehydrogenase. In total, 20 µL MTT (5 mg/mL in phosphate buffered saline) was added to each well 4 h prior to the desired endpoint to dissolve the formazan

crystals. The absorbance, which represented the Optical Density (OD), was measured at 570 nm in a 96-well plate reader and analyzed statistically to draw the growth inhibition curve, calculate the Inhibition Rate (IR).

**Morphology of apoptotic cells:** Hl60 cells were plated in 24-well plates (2×10<sup>5</sup> cells/well) during the logarithmic phase. After 6 h, Rg3 (4.0 µg/mL), cytarabine (10.0 µg/mL) and Rg3 (4.0 µg/mL)+cytarabine (10.0 µg/mL) were added and then incubated for 48 h. Then, the cells were transferred into a 1.5 mL tube. The supernatant was removed after centrifugation for 5 min at 2,000 rpm. The cells were stained for 10 min. Then hl60 cells were washed with PBS, stained and fixed with 0.2% crystal violet in 10% phosphate-buffered formaldehyde for 10 min. Crystal violet solution was then discarded and cells were washed gently with water. Cells were observed and photographed under a microscope.

**Apoptosis assay using flow cytometry:** Hl60 cells during the logarithmic phase were plated in 24-well plates (2×10<sup>5</sup> cells/well). After 6 h, Rg3 (4.0 µg/mL), cytarabine (10.0 µg/mL) and Rg3 (4.0 µg/mL)+cytarabine (10.0 µg/mL) were added. Each concentration were performed three parallel wells. After treatment for 48 h, floating cells in the medium and hl60 cells were collected. Then hl60 cells were washed with PBS and adjust the cell concentration to 1×10<sup>6</sup>/mL. Cells were stained with Propidium Iodide (PI) according to manufacturer's instructions. Untreated cells were used as the control for PI staining. Cells were analyzed immediately after staining using a flow cytometer. The DNA content of cells were detected with sub-G1 peak of cells counted as apoptotic cells and apoptosis rate of different group cells were calculated.

**Western blot analysis:** Cultured cells were washed twice with 1×PBS, followed by the addition of 1 mL of PBS and cells were removed into a cold Eppendorf tube. Cells were homogenized with lysis buffer and centrifuged for 1 h. The protein concentration was measured by the Bradford method and equal amount of proteins were separated on an SDS/1%-polyacrylamide gel and then transferred to a nitrocellulose membrane. Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline solution. The membranes were immunoblotted with primary specific antibodies: rabbit polyclonal for Bax, Bcl-2. The blot was then incubated with the corresponding conjugated anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase. Immunoreactive proteins were detected and the relative density of the protein bands was scanned.

**Statistical analysis:** Statistical analysis was performed using SPSS version 13.0 software. Data are expressed

as mean±standard deviation and  $p<0.05$  was considered to indicate a statistically significant difference.

## RESULTS AND DISCUSSION

**Anti-proliferation effect of Rg3 and cytarabine:** HL60 cells were treated separately with 2.0, 4.0, 8.0 and 16.0  $\mu\text{g/mL}$  Rg3 and cell viability was observed by MTT assay after 48 h. Cell viabilities were 100.3, 95.1, 89.5 and 83.2%. The cells treated with 2.0  $\mu\text{g/mL}$  Rg3 showed no significant difference compared with the control group, but a significant decrease of cell viability in the treated with 4.0  $\mu\text{g/mL}$ . HL60 cells were treated separately with 5, 10, 20, 40  $\mu\text{g/mL}$  cytarabine for 48 h. There was a significant decrease of cell viability in all those treated. Cell viabilities were 92.8, 86.3, 72.4 and 63.6%, respectively. These results suggest that Rg3 (4.0  $\mu\text{g/mL}$ ) and cytarabine (5-40  $\mu\text{g/mL}$ ) can inhibit the cell growth of hl60 human acute leukemia cells. For the groups of combination of Rg3 and cytarabine, hl60 cells were treated with 4.0  $\mu\text{g/mL}$  Rg3+ 5, 10, 20, 40  $\mu\text{g/mL}$  cytarabine for 48 h, the cell viabilities were 81.6, 72.5, 64.9 and 58.9%, respectively. Compared with the cytarabine (5, 10, 20, 40  $\mu\text{g/mL}$ ) group, the cell proliferation of the cytarabine combined with Rg3 (4.0  $\mu\text{g/mL}$ ) group was decreased significantly ( $p<0.001$ ). These results suggesting that Rg3 can enhance the anti-cancer effects of the chemotherapeutic agent cytarabine as shown in Fig. 2.

**Cell morphology observation:** To test whether the increase in cell inhibity observed after treatment with cytarabine and/or Rg3 was due to apoptosis, hl60 cells were stained with dye after exposure to the treatments for 48 h. HL60 cells of control group can be observed in high density, which were big, round, strong refraction and little granules in cytoplasmic (Fig. 3a). Compared with the control group, hl60 cells treated with cytarabine (10  $\mu\text{g/mL}$ ) and/or Rg3 (4.0  $\mu\text{g/mL}$ ) were in poor growth (Fig. 3b and c). The cells were smaller, irregular and decrease in number. As shown in Fig. 3, typical apoptotic nuclearmorphology with nuclear shrinkage, DNA condensation and fragmentation was presented in the cytarabine and cytarabine+Rg3-treated cells, but not in the non-treated controls group. The number of cells following treatment with the combination of cytarabine and Rg3 decreased remarkably and cell aspects are different compared with the treatment group of cytarabine alone. These results showed that the combined use of cytarabine and Rg3 could inhibit the growth of hl60 human acute leukemia cells more effectively than the use of cytarabine alone.

**Detection of apoptosis:** We thus evaluated apoptotic cell death induced by the combination of Rg3 and cytarabine. In this study, activities of cytarabine and Rg3 were evaluated by flow cytometry after staining

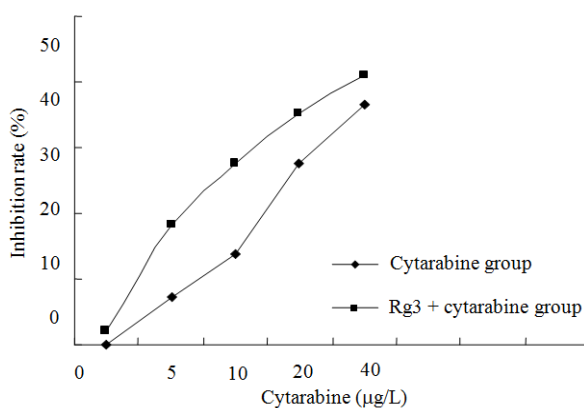


Fig. 2: Ginseng extract and cytarabine on the cell inhibition rate of hl60 cells

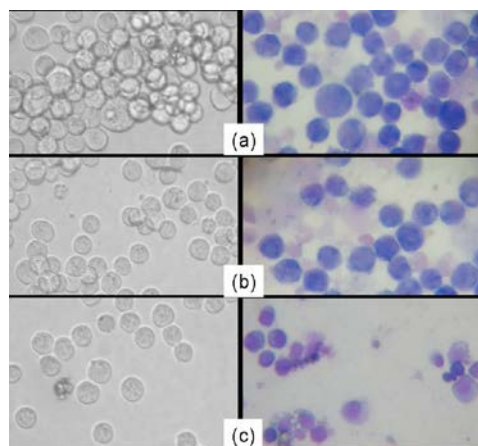


Fig. 3: Morphological changes of hl60 cells ( $\times 250$ ); (a): control group; (b): cytarabine (10  $\mu\text{g/mL}$ ) group; (c): cytarabine (10  $\mu\text{g/mL}$ )+Rg (4.0  $\mu\text{g/mL}$ ) group

Table 1: Induction of apoptosis by cytarabine and the combination of cytarabine and Rg3

Group	Apoptosis rate (%)
Cytarabine (20 $\mu\text{g/mL}$ )	23.41±0.62
Rg3 (4.0 $\mu\text{g/mL}$ )+cytarabine (20 $\mu\text{g/mL}$ )	38.54±0.85*
Cytarabine (40 $\mu\text{g/mL}$ )	39.66±0.97
Rg3 (4.0 $\mu\text{g/mL}$ )+cytarabine (40 $\mu\text{g/mL}$ )	45.28±0.75*

Compared with cytarabine group, \*  $p<0.01$

with PI. The apoptosis induced by exposure to cytarabine and Rg3 for 48 h was concentration-dependent. Significant increase were observed. Apoptotic cells, including those both in early and late apoptosis, induced by Rg3 (4.0  $\mu\text{g/mL}$ ) combined with cytarabine (5, 10, 20, 40  $\mu\text{g/mL}$ ) were significantly increased compared with cytarabine (5, 10, 20, 40  $\mu\text{g/mL}$ ) alone ( $p<0.01$ ). As shown in Table 1, apoptosis induced by the combination of cytarabine and Rg3 was mainly due to the presence of Rg3. These results suggesting that Rg3 can enhance the anti-cancer effects of the chemotherapeutic agent cytarabine by promoting apoptosis.

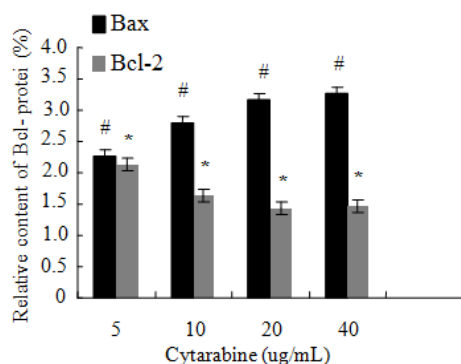


Fig. 4: Effect of the combination treatment of Rg3 and cytarabine on expression of apoptosis regulatory proteins; Note: compared with cytarabine group: \*: Bcl-2 protein,  $p < 0.05$ ; #: Bax protein,  $p < 0.01$

**Expression of apoptotic regulatory proteins:** To identify the mechanism underlying the enhanced apoptotic response to Rg3 and cytarabine combination treatment, we next assessed the expression of pro and anti-apoptotic proteins by western blotting. H160 cells were treated with Rg3 (4.0 µg/mL) combined with cytarabine (5, 10, 20, 40 µg/mL) and cytarabine (5, 10, 20, 40 µg/mL) alone for 48 h and whole-cell extracts were subjected to western blotting. Combination treatment substantially inhibited levels of Bcl-2, but the expression of pro-apoptotic proteins Bax was significantly increased compared to expression after treatment with cytarabine alone. Using the image analysis system for quantitative analysis, the significant differences are showed between the Rg3 combined with cytarabine group and cytarabine group (Fig. 4). Therefore, we think ginseng extract enhances anti-cancer effect through regulating of apoptotic cell death (Yuan *et al.*, 2010).

## CONCLUSION

The main findings of the present study are that ginseng extract Rg3 significantly arguments cell growth inhibition and induction of apoptosis by chemotherapeutic agents cytarabine in acute leukemia. The interplay of herbal medicine and human health has been documented for thousands of years. Ginseng is one of the most commonly used herbal medicines and is reported to have a wide range of pharmacological and therapeutic applications (Jian *et al.*, 2009). It has previously been demonstrated that ginsenosides, the major pharmacologically active ingredients of ginseng, possess MDR-modulating activity (Sung *et al.*, 2014). Ginsenoside potentiates the effects of anticancer agents in multidrug resistant cells.

In our current study, the ginseng extract Rg3 showed a stronger anti-proliferation effect on acute leukemia cells. Although cytarabine has certain anti-

cancer effects on acute leukemia cells, the present study showed that even when the treatment concentration of acute leukemia was increased to high doses, cell proliferation was decreased slowly. This result is similar to that observed in clinical studies, where the anti-cancer effect is not increased significantly by high doses of cytarabine although they lead to severe side effects. Decreasing the dose of chemotherapy and increasing the anti-cancer effect by combining cytarabine with other medicines are important considerations (Xu *et al.*, 2007). In this study, the anti-proliferation effect of Rg3 combined with cytarabine was investigated. Treated with combination of cytarabine (10 µg/mL) and Rg3 (4.0 µg/mL) for 48 h, the cell viabilities were 72.5%. This is lower than the cell viabilities observed with treatment with cytarabine 10 µg/mL (86.3%), suggesting that combining cytarabine with Rg3 can reduce the dose of cytarabine needed and significantly increasing the anti-proliferation effect on h160 cells. Since it is well known that cytarabine has cytotoxic effects, this synergistic effect between Rg3 and cytarabine makes it possible to reduce the dose of cytarabine in chemotherapy and thereby further decrease dose-related toxicity. In this study, a very strong apoptotic effect was observed after treatment with a combination of Rg3 and cytarabine different from the single use of cytarabine. Thus, it is suggested that the apoptotic effect of the combined treatment was mainly contributed by Rg3 (Wang *et al.*, 2009).

The use of complementary and alternative medicine including botanical extracts is becoming increasingly popular among cancer patients (Takimoto and Awada, 2008). The main reason for using botanical products has been to boost the immune system, decrease chemotherapy or radiotherapy-induced side effects and improve quality of life. In this study, Rg3, a chemical component extracted from Panax ginseng, was found to have a synergistic anti-proliferation effect on h160 human acute leukemia cells.

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