

# Notoginseng enhances anti-cancer effect of 5-fluorouracil on human colorectal cancer cells

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## Abstract

**Purpose** *Panax notoginseng* is a commonly used Chinese herb. Although a few studies have found that notoginseng shows anti-tumor effects, the effect of this herb on colorectal cancer cells has not been investigated. 5-Fluorouracil (5-FU) is a chemotherapeutic agent for the treatment of colorectal cancer that interferes with the growth of cancer cells. However, this compound has serious side effects at high doses. In this study, using HCT-116 human colorectal cancer cell line, we investigated the possible synergistic anti-cancer effects between notoginseng flower extract (NGF) and 5-FU on colon cancer cells.

**Methods** The anti-proliferation activity of these modes of treatment was evaluated by MTS cell proliferation assay. Apoptotic effects were analyzed by using Hoechst 33258 staining and Annexin-V/PI staining assays. The anti-proliferation effects of four major single compounds from NGF, ginsenosides Rb1, Rb3, Rc and Rg3 were also analyzed.

**Results** Both 5-FU and NGF inhibited proliferation of HCT-116 cells. With increasing doses of 5-FU, the anti-proliferation effect was slowly increased. The combined usage of 5-FU 5  $\mu$ M and NGF 0.25 mg/ml, significantly increased the anti-proliferation effect (59.4  $\pm$  3.3%) compared with using the two medicines separately (5-FU 5  $\mu$ M, 31.1  $\pm$  0.4%; NGF 0.25 mg/ml, 25.3  $\pm$  3.6%). Apoptotic analysis showed that at this concentration, 5-FU did not exert an apoptotic effect, while apoptotic cells induced by NGF were observed, suggesting that the anti-proliferation target(s) of NGF may be different from that of 5-FU, which is known to inhibit thymidilate synthase.

**Conclusions** This study demonstrates that NGF can enhance the anti-proliferation effect of 5-FU on HCT-116 human colorectal cancer cells and may decrease the dosage of 5-FU needed for colorectal cancer treatment.

**Keywords** *Panax notoginseng* · 5-Fluorouracil · Chemotherapy · HCT-116 human colorectal cancer cells · Anti-proliferation · Apoptosis

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## Introduction

Human colorectal cancer is a significant public health problem in the western world and is a common condi-

tion that affects about 6% of the population [9]. In the United States, this cancer is the second-leading cause of cancer-related deaths, and the second most prevalent cancer worldwide [10, 12]. Although many early stage colorectal cancers are cured by surgical resection alone, most often surgery is combined with adjuvant radio- and chemotherapy. Radiotherapy is often combined with one or more chemotherapeutic agents. However, chemotherapies are still limited by severe side effects and dose-limiting toxicity. The drug-related adverse events not only worsen patients' quality of life, but can also lead to their refusal to continue the potentially curative chemotherapy [23]. There are several human colorectal cancer cell lines, of which HCT-116 is widely used in laboratory cancer research [19], and has been a model for the cellular pathways studies of chemotherapy on cancer cells [4]. We selected HCT-116 cell line for this study.

5-Fluorouracil (5-FU) is a chemotherapy drug that interferes with the growth of cancer cells. It can be used to treat many types of cancers, including cancer of the colon, rectum, breast, stomach, head, and neck. 5-Fluorouracil is the commonly used drug in the treatment of colorectal cancer, especially metastatic colorectal cancer. However, this drug has serious side effects including nausea, fatigue, and a decrease in the number of blood cells [7]. Studies have found that for the treatment of metastatic colon cancer, higher doses of 5-FU are no more effective yet increase side effects [18].

Currently, newer chemotherapeutic agents continue to be investigated, including those derived from botanical sources [11]. Patients with cancer often resort to complementary and alternative medical means to treat the side effects of chemotherapy [21]. We have investigated the effects of botanical extracts on reducing chemotherapeutic side effects and found that American ginseng berry can attenuate cisplatin-induced nausea and vomiting [17]. Additionally, the extract from American ginseng enhanced the anti-proliferation effect of cisplatin on human breast cancer cells, suggesting that it possesses its own anti-cancer effect [1].

Notoginseng (*Panax notoginseng*) and American ginseng (*Panax quinquefolius*) are different species, but belong to the same genus (genus *Panax*). Distributed throughout the southwest of China, Burma, and Nepal, notoginseng is a remedy that has a long history of use in China and other Asian countries. The earliest scientific description of notoginseng was in *Materia Medica*, a dictionary of Chinese herbs, written by Li Shi Zhen (1518–1593 AD). The portion of the plant commonly used in remedies is the root, which is

dug up before the plant flowers or after the fruit has ripened. In addition, other plant parts of notoginseng, rootlet, leaf and flower, are also used and the activity and safety are similar to that of the root [6]. In traditional Chinese herbal medicine, notoginseng is regarded as the emperor herb in the treatment of different types of wounds because it is a favorite medicine for both internal and external bleeding. Recent studies found that notoginseng showed anti-tumor effects [14, 15]. However, the effect of this herb on colorectal cancer cells has not been evaluated. Based on our screening data, the anti-cancer effect of notoginseng flower extract (NGF) is stronger than that of the root extract. In this study, we investigated the anti-proliferation effects of NGF and/or 5-FU on HCT-116 human colorectal cancer cells. The notoginseng and/or 5-FU induced cancer cell apoptosis were also evaluated.

## Materials and methods

### Chemicals

All cell culture plasticware were purchased from Falcon Labware (Franklin Lakes, NJ), and Techno Plastic Products (Trasadingen, Switzerland). Trypsin, McCoy's 5A medium, and PBS were obtained from Mediatech, Inc. (Herndon, VA). 5-Fluorouracil was obtained from American Pharmaceutical Partners Inc. (Schaumburg, IL). Hoechst 33258, formaldehyde, NP40, crystal violet, and penicillin/streptomycin were obtained from Sigma (St. Louis, MO). MTS assay kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was obtained from Promega (Madison, WI). Annexin V-FITC Apoptosis Detection Kit was obtained from BD Biosciences (Rockville, MD). Ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Rg3 and notoginsenoside R1 were obtained from Delta Information Center for Natural Organic Compounds (Xuancheng, AH, China). HPLC grade methanol, *n*-butanol, acetonitrile, and absolute ethanol were obtained from Fisher Scientific (Pittsburgh, PA). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA).

### Plant materials

The most commonly used notoginseng variety is cultivated commercially in several provinces of southwest China. The chemical composition of notoginseng could be different based on its origin. We selected *P. notoginseng* cultivated in Wenshan for our study since the quality of notoginseng from Wenshan has been controlled

under the Good Agricultural Practice criteria [5], and has been frequently used in many previous studies [8, 26]. This notoginseng was obtained from Yunnan Chinese Herbal Medicine Company and the root and flower were collected in 2004 and was authenticated by the Department of Pharmacognosy, China Pharmaceutical University, Nanjing. The sample was identified by macro-morphological and microscopic characteristics, and thin layer chromatography (TLC) based on Chinese Pharmacopoeia [22] and it was identified that samples were the root and flower of *Panax notoginseng* (Burk.) F. H. Chen.

#### Preparation of notoginseng extracts

The notoginseng sample was ground to powder and passed through a 40 mesh. Twenty-five gram of notoginseng powder was extracted with 500 ml 75% ethanol for 4 h, the water bath was maintained at 90°C. When the solution was cooled, it was filtered with P8 filter paper (Fisher Scientific, Pittsburgh, PA) and the filtrate was collected. The residue was extracted with 500 ml 75% ethanol once more and then filtered while the solution was cooled. The filtrate was mixed and the solvent was evaporated under vacuum. The dried extract was dissolved in 100 ml water then extracted with water-saturated *n*-butanol. The *n*-butanol phase was evaporated under vacuum, and then lyophilized. Notoginseng root and flower extracts were stored in -20°C freezer before use.

#### High performance liquid chromatography (HPLC) analysis

The HPLC system was a Waters 2960 instrument (Milford, MA), with a quaternary pump, automatic injector, a photodiode array detector (Model 996), and Waters Millennium<sup>32</sup> software for peak identification and integration. The separation was carried out on an Alltech Ultrasphere C18 column (5  $\mu$ , 250  $\times$  3.2 mm I.D.) (Deerfield, IL) with a guard column (Alltech Ultrasphere C18, 5  $\mu$ , 7.5  $\times$  3.2 mm I.D.). For HPLC analysis, a 20  $\mu$ l sample was injected into the column and eluted at room temperature with a constant flow rate of 1.0 ml/min. Acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 18% solvent A and 82% solvent B, changed to 21% A for 20 min, then changed to 26% A for 3 min and held for 19 min; changed to 36% A for 13 min; changed to 50% A for 9 min; changed to 95% A for 2 min and held for 3 min; changed to 18% A for 3 min and held for 8 min. The detection wavelength was set to 202 nm. Ginsenosides and notoginsenoside standards, and

notoginseng extracts were dissolved in methanol. All solutions were filtered through Millex 0.2  $\mu$ m nylon membrane syringe filters (Millipore Co., Bedford, MA) before use.

#### Cell culture

HCT-116 human colorectal cancer cells were obtained from American Type Culture Collection (Manassas, VA). IEC-18 rat intestinal epithelial cells were obtained from Dr. Kenneth A. Drabik in Department of Medicine, University of Chicago. The HCT-116 cells were cultured with McCoy's 5A containing 10% PBS, 50 IU penicillin/streptomycin, and IEC-18 cells were cultured with DMEM medium containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 4.5 g/l glucose supplemented with 5% FBS and 50 IU penicillin/streptomycin, in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

#### Cell proliferation analysis

Notoginseng flower extract was dissolved in 50% ethanol. The 5-FU, 50 mg/ml, was diluted with milli Q water. NGF and 5-FU stock solutions were stored at 4°C before use. Cells were seeded in 96-well plates (2  $\times$  10<sup>4</sup> cells/well). After 24 h, various concentrations of NGF and/or 5-FU were added to the wells. The final concentration of ethanol in the NGF group was 0.5%. Controls were exposed to culture medium containing the same quantity of ethanol without drugs. All experiments were performed in triplicates and repeated two or three times. The treatment process of notoginseng root extract was similar to that of NGF.

After treatment with NGF and/or 5-FU, cell growth was evaluated using an MTS assay according to the manufacturer's instructions. Briefly, at the end of the incubation period, the medium was replaced with 100  $\mu$ l of fresh medium, 20  $\mu$ l of MTS reagent in each well, and the plate was returned to incubator for 1–2 h. A 60  $\mu$ l aliquot of medium from each well was transferred to an ELISA 96-well plate and its absorbance at 490 nm was recorded.

#### Crystal violet cell viability staining assay

Cells were seeded in 24-well plates (1  $\times$  10<sup>5</sup> cells/well). After 24 h, NGF (0.25 mg/ml), 5-FU (5  $\mu$ M) and NGF (0.25 mg/ml) + 5-FU (5  $\mu$ M) were added and then incubated for 48 h. Then medium was discarded and adherent cells were washed with PBS, stained and fixed with 0.2% crystal violet in 10% phosphate-buffered formaldehyde for 2 min. Crystal violet solution was then

discarded and cells were washed gently with water. Adherent cells were observed and photographed under a microscope.

#### Hoechst 33258 staining assay

Cells ( $1 \times 10^5$  cells/well in 24 well-plate) were treated with NGF (0.25 mg/ml), 5-FU (5  $\mu$ M) and NGF (0.25 mg/ml) + 5-FU (5  $\mu$ M) for 48 h. Then, the cells were trypsinized and 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 in the dark with the solution containing 0.01 mg/ml Hoechst 33258, 33 mg/ml of formaldehyde, and 5 mg/ml of NP-40 in PBS. The cells were then examined using a fluorescence microscope with an excitation wavelength of 365 nm.

#### Apoptosis assay using flow cytometry

Cells were seeded in 24-well tissue culture plates ( $2 \times 10^5$ ). After culturing for 2 days, medium was replaced with fresh medium and NGF (1 mg/ml), 5-FU (20  $\mu$ M) and NGF (1 mg/ml) + 5-FU (20  $\mu$ M) were added. After treatment for 8 h, floating cells in the medium and adherent cells were collected. Using Annexin V-FITC Apoptosis Detection Kit (Rockville, MD), cells were stained with Annexin-V FITC and propidium iodide (PI) according to manufacturer's instructions. Untreated cells were used as the control for double staining. Cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and the Flojo software (Ashland, OR). For each measurement, at least 20,000 cells were counted.

#### Anti-proliferation effect of ginsenosides Rb1, Rb3, Rc and Rg3

The anti-proliferation effects of four single compounds in NGF were assayed. Ginsenosides Rb1, Rb3, Rc and Rg3 were dissolved in 50% ethanol. Cells were seeded in 96-well plates ( $2 \times 10^4$  cells/well). Controls were exposed to culture medium containing the same quantity of ethanol (0.5%) without drugs. The treatment time was 48 h and the cell proliferation analysis process was similar with that of NGF.

#### Statistical analysis

Data were presented as mean  $\pm$  standard error (SE). A one-way ANOVA was employed to determine whether the results had statistical significance. In some cases, Student's *t*-test was used for comparing

two groups. The level of statistical significance was set at  $P < 0.05$ .

## Results

#### High performance liquid chromatography analysis of NGF

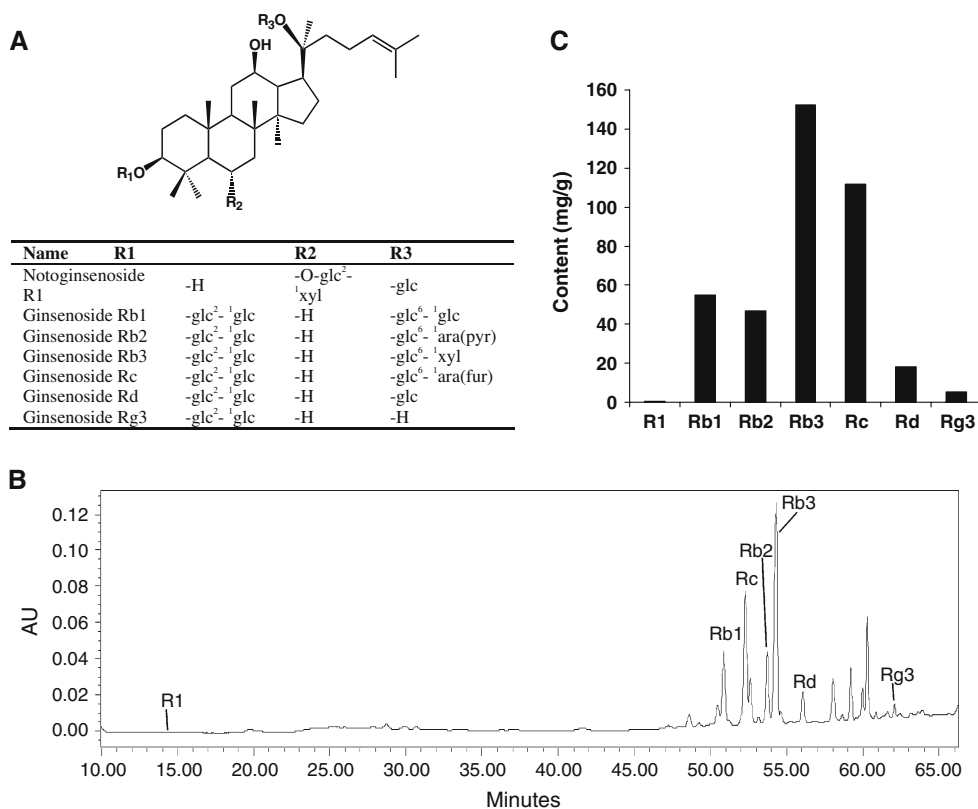
The chemical structures of seven saponins are shown in Fig. 1a. Based on the HPLC procedures described previously, reverse-phase HPLC appeared efficient for the qualitative and quantitative determination of notoginseng saponins. The calibration curves of standards were established using peak area and their related amount of selected saponins. For all linearity curves, the correlation coefficients were no less than 0.999. Notoginseng saponins in the extract were identified by comparing the retention times and UV spectrum of authentic ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Rg3 and notoginsenoside R1 standards obtained from the mixed standards' chromatograms. The HPLC chromatogram of NGF is shown in Fig. 1b. The results of the analysis, shown in Fig. 1c, indicate that the main constituents in NGF were ginsenoside Rb1, Rb2, Rb3 and Rc. Ginsenoside Rb3 was present in the highest concentration in NGF (15.2%). Ginsenoside Rd and Rg3 were in lower concentrations, and notoginsenoside R1 was detected as a trace saponin in NGF.

#### Anti-proliferation effect of NGF and 5-FU

We evaluated the anti-proliferation effect using extracts from the root and flower of notoginseng on HCT-116 human colorectal cancer cells. After treatment for 48 h, the cell proliferation of the group receiving notoginseng root extract was  $98.1 \pm 1.6\%$  at 0.1 mg/ml, and  $81.7 \pm 2.3\%$  at 0.25 mg/ml; while the effect of NGF was  $95.5 \pm 2.9\%$  at 0.1 mg/ml, and  $75.2 \pm 3.6\%$  at 0.25 mg/ml. The flower extract showed more potency than that of the root extract of notoginseng. Thus, we selected NGF for the following studies.

For the time and dose dependent tests, HCT-116 cells were treated separately with 0.1 and 0.25 mg/ml NGF and observed at the intervals of 12, 24, 48, 72 and 96 h followed by MTS assay for cell viability. The cells treated with 0.1 mg/ml NGF showed no significant difference compared with the control group. There was a significant decrease of cell viability in those treated with 0.25 mg/ml after 48 h. Under the treatment of 0.1 and 0.25 mg/ml NGF, cell viability was 101.7 and 91.5% after 12 h; 103.8 and 89.1% after 24 h; 95.5 and

**Fig. 1** Chemical structures and HPLC analysis of ginsenosides in notoginseng flower extract. The structures of determined ginsenosides are shown in **a**. HPLC chromatogram of notoginseng extract recorded at 202 nm is shown in **b**. HPLC conditions are described in **Materials and methods**. Saponin content of selected ginsenosides is shown in **c**. The results showed that the main constituents of notoginseng flower extract are ginsenoside Rb3 and Rc, while notoginsenoside R1 has limited content in this extract



75.2% after 48 h; 101.7 and 72.4% after 72 h; 103.0 and 77.8% after 96 h, respectively (Fig. 2a).

Since the anti-cancer effect of 5-FU has been extensively studied previously, the treatment time of 5-FU was selected as 48 h. HCT-116 cells were treated separately with 2.5, 5, 20, 50, and 100  $\mu$ M of 5-FU for 48 h. There was a significant decrease of cell viability in all those treated. Cell viabilities were 83.5, 67.7, 61.3, 52.9, and 49.5%, respectively (Fig. 2b).

A rat intestinal epithelial cell line, IEC-18 cells, was used to evaluate the safety of NGF. Figure 2c shows the activities of NGF on the proliferation of IEC-18 cells. At the dose of 0.1 and 0.25 mg/ml, NGF did not inhibit the cell growth. In comparison with the control (100%), the cell viabilities of NGF on IEC-18 cells are 107.3% at 0.1 mg/ml, and 99.1% at 0.25 mg/ml, respectively.

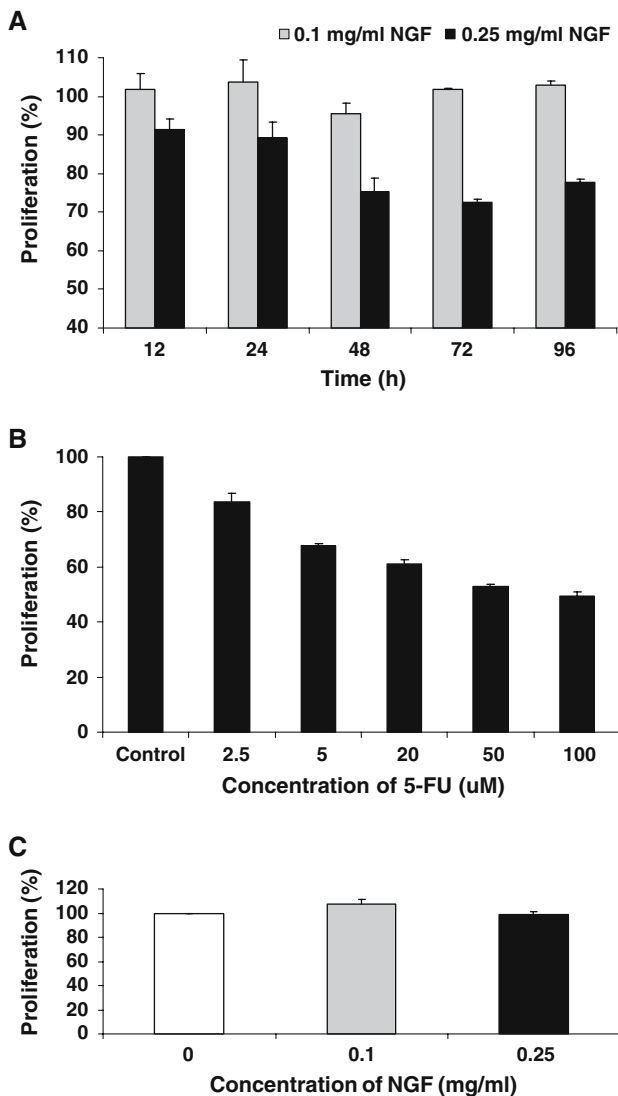
These results suggest that NGF (0.25 mg/ml) and 5-FU (2.5–100  $\mu$ M) can inhibit the cell growth of HCT-116 human colorectal cancer cells. NGF (0.25 mg/ml) does not inhibit the growth of IEC-18 rat intestinal epithelial cells.

#### Effect of combined treatment of NGF and 5-FU on HCT-116 cells

HCT-116 cells were treated separately with 0.1, 0.25 mg/ml NGF, 5  $\mu$ M 5-FU, 5  $\mu$ M 5-FU + 0.1 mg/ml

NGF, and 5  $\mu$ M 5-FU + 0.25 mg/ml NGF for 48 h. For the groups of combination of 5-FU and NGF, the cell proliferations were decreased compared with only the use of 5-FU 5  $\mu$ M. Treated with 0.1 mg/ml NGF, 0.25 mg/ml NGF, and 5  $\mu$ M 5-FU, cell viabilities were 94.3, 74.7, and 68.9%. For the treatment groups combining 5-FU 5  $\mu$ M with 0.1 mg/ml NGF, and 5-FU 5  $\mu$ M with 0.25 mg/ml NGF, the cell viabilities were 60.4 and 40.6%, respectively (Fig. 3a). Compared with the 5  $\mu$ M 5-FU group, the cell proliferation of the 5-FU (5  $\mu$ M) combined with NGF 0.25 mg/ml group was decreased significantly. Moreover, the anti-proliferation effect of this combination group (59.4%) was stronger than that of the 100  $\mu$ M 5-FU group (50.5%), suggesting that NGF can enhance the anti-cancer effects of the chemotherapeutic agent 5-FU.

Morphological observation was conducted after crystal violet staining. Figure 3b shows the morphological characteristics after treatment for 48 h. For the control group, cells were smaller, compacted and staining was even (Fig. 3b, panel 1). The 5-FU 5  $\mu$ M treatment group showed more dead cells, and the cell confluence was not as high as the control group (Fig. 3b, panel 3). A similar result was observed for the NGF 0.25 mg/ml treatment group, and in addition, some cells contained small vacuoles (Fig. 3b, panel 2). For the combination



**Fig. 2** Notoginseng flower extract and 5-fluorouracil on the cell proliferation of HCT-116 human colorectal cancer cells (**a**, **b**) and IEC-18 rat intestinal epithelial cells (**c**) determined by MTS method. Time and dose dependent effect of NGF on HCT-116 cells is shown in **a**. After treatment with NGF (0.1, 0.25 mg/ml), cell proliferation was initially measured at 12, 24, 48, 72 and 96 h. After 48 h treatment, the proliferation was stable. The effect of 5-FU on HCT-116 cells is shown in **b**. Cell proliferation in the presence of 5-FU (2.5–100 µM) was measured after 48 h treatment. High dose of 5-FU (100 µM) could not increase its anti-proliferation effect on HCT-116 cells. The effect of NGF on IEC-18 cells (treatment for 48 h) is shown in **c** (mean  $\pm$  SE,  $n = 3$ ). 5-FU 5-fluorouracil, NGF notoginseng flower extract

of 5-FU 5 µM and NGF 0.25 mg/ml group, cell confluence was much lower than that of control, and small vacuoles were also observed in cells (Fig. 3b, panel 4). These results showed that the combined use of 5-FU and NGF could inhibit the growth of HCT-116 human colorectal cancer cells more effectively than the use of 5-FU or NGF alone.

### Induction of apoptosis by NGF and the combination of 5-FU and NGF

To test whether the decrease in cell viability observed after treatment with 5-FU and/or NGF was due to apoptosis, HCT-116 cells were stained with Hoechst 33258 dye after exposure to the treatments for 48 h. The dye stains condensed chromatin of apoptotic cells more brightly than chromatin of normal cells. As shown in Fig. 4a, Hoechst staining, which correlates with the presence of cells with typical apoptotic nuclear morphology (nuclear shrinkage, DNA condensation and fragmentation), was present in the NGF and 5-FU + NGF-treated cells (Fig. 4a, panel 2, 4), but not in the non-treated controls (Fig. 4a, panel 1) and the 5-FU 5 µM treatment group (Fig. 4a, panel 3).

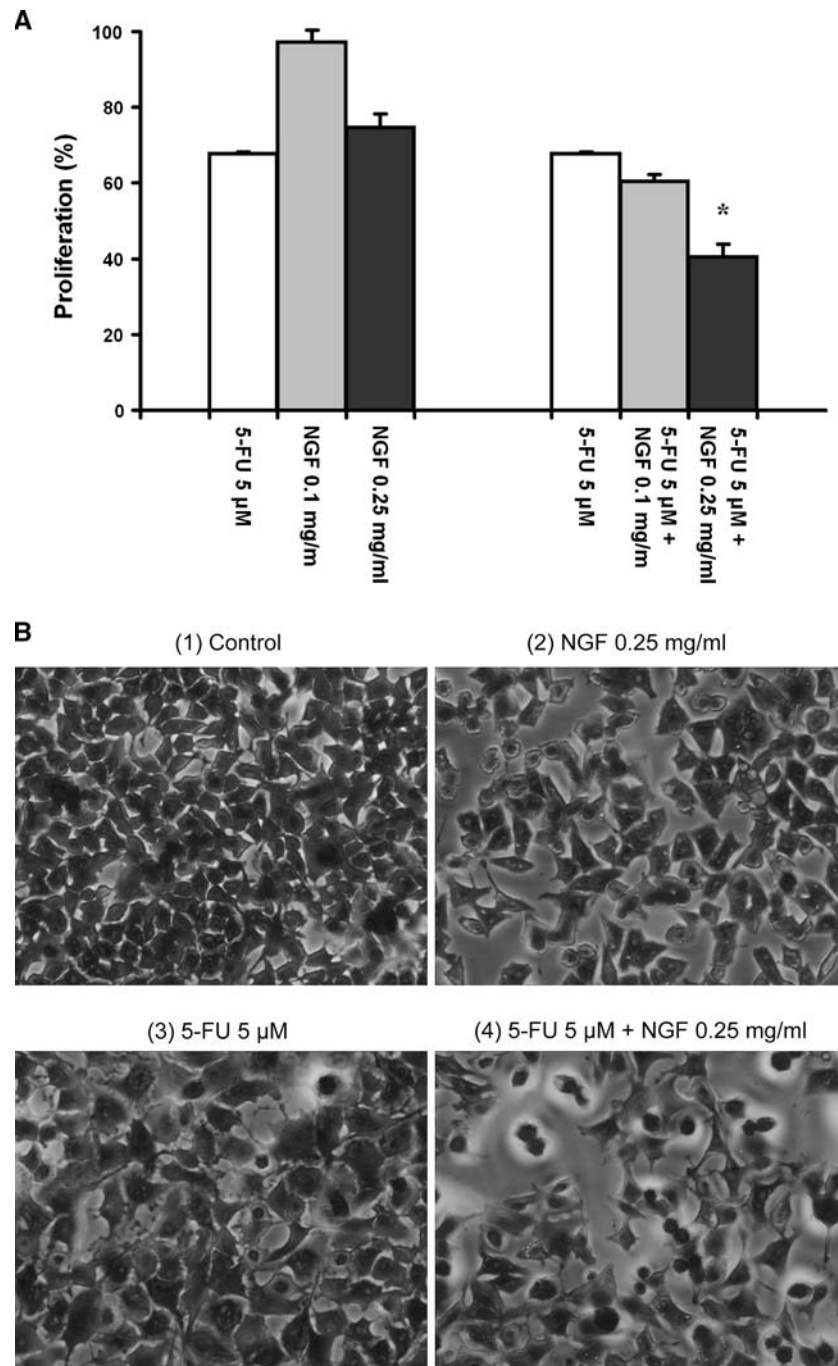
Induction of apoptosis was confirmed by flow cytometry after staining with Annexin V/PI. The cytogram in Fig. 4b shows a bivariate Annexin V/PI analysis of HCT-116 cells. Viable cells were negative for both PI and Annexin V (lower left quadrant), early apoptotic cells were positive for Annexin V and negative for PI (lower right quadrant) whereas late apoptotic cells displayed both positive for Annexin V and PI (upper right quadrant). Non-viable cells which, underwent necrosis, were positive for PI and negative for Annexin V (upper left quadrant). In this study, acute activities of NGF and/or 5-FU were evaluated. Since the treatment time was 8 h, 1/6 of treatment for cell proliferation tests (48 h), the dose of NGF and/or 5-FU was increased by four times compared with the proliferation tests. Apoptotic cells, including those both in early and late apoptosis, induced by NGF 1 mg/ml combined with 5-FU 20 µM (41.3%, Fig. 4b, panel 4) were similar to those of the NGF 1 mg/ml group (39.0%, Fig. 4b, panel 2). It is clear from this cytogram that NGF induced massive apoptosis and most of these cells were already at the late apoptotic stage at 8 h. Nevertheless, treatment with 5-FU 20 µM did not induce any increase in apoptosis (0.8%, Fig. 4b, panel 3) and were essentially similar to control cells (0.8%, Fig. 4b, panel 1). These results suggest that the apoptosis induced by the combination of 5-FU and NGF was mainly due to the presence of NGF.

### The effect of four single compounds in NGF on HCT-116 cells

The pharmacological effect of herbal medicine is based on its constituents. It is well known that saponins are active compounds in notoginseng [32]. The results

**Fig. 3** Effects of 5-fluorouracil combined with notoginseng flower extract on the growth of HCT-116 human colorectal cancer cells. HCT-116 cells were exposed to 5-FU and/or NGF for 48 h and cell proliferation was determined by MTS (**a**) (mean  $\pm$  SE,  $n = 3$ ).

\* $P < 0.05$ , compared to 5-FU alone. The data showed that NGF 0.25 mg/ml could enhance the anti-proliferation effect of 5-FU 5  $\mu$ M on HCT-116 cells significantly. The morphological aspects were photographed with a microscope after staining with crystal violet (**b**). The number of cells following treatment with the combination of 5-FU and NGF decreased remarkably and cell aspects are different compared with the treatment groups of 5-FU or NGF alone. 5-FU 5-fluorouracil, NGF notoginseng flower extract

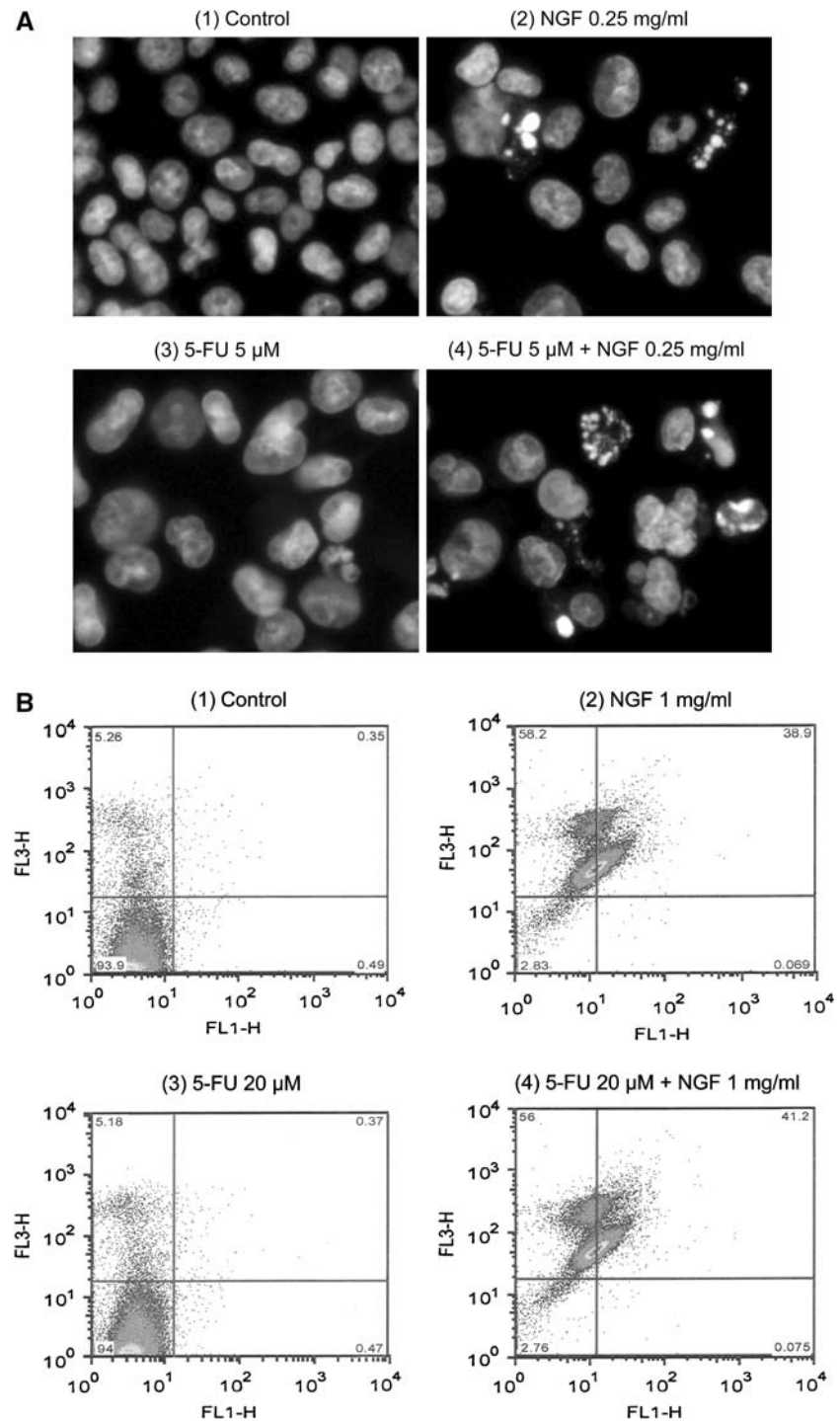


from HPLC show that there are several saponins in NGF (Fig. 1). We determined the anti-proliferation effects of four representative saponins from NGF on HCT-116 cells. At the concentration of 30–300  $\mu$ M, ginsenoside Rb1, Rb3 and Rc did not show anti-proliferation activity, while ginsenoside Rg3 can inhibit the growth of HCT-116 cells (Fig. 5). At the concentration of 300  $\mu$ M, the anti-proliferation rate of Rg3 was  $97.9 \pm 0.8\%$ , suggested that ginsenoside Rg3 may be an active constituent in NGF.

## Discussion

The interplay of herbal medicine and human health has been documented for thousands of years. Although the commonly used plant part of notoginseng is the root, other plant parts including the flower has also been used as herbal medicine. In traditional Chinese medicine [6], the activity of notoginseng flower is described as: “it cools heat, resolves toxicity, and calms the liver. It is used for dizziness, vertigo, and tinnitus associated

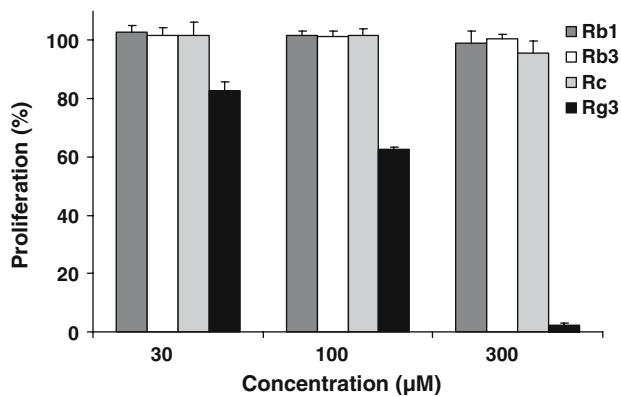
**Fig. 4** Apoptosis assay of HCT-116 human colorectal cancer cells using Hoechst 33258 and Annexin V/PI staining. For the Hoechst 33258 staining assay (a), HCT-116 cells were incubated for 48 h with 5-FU and/or notoginseng flower extract (NGF). Some apoptotic cells were found in the NGF and the 5-FU + NGF groups. For the flow cytometry analysis (b), HCT-116 cells were incubated for 8 h with 5-FU and/or NGF and flow cytometry was performed after staining with Annexin V/PI. Compared with the control (apoptotic cells <1%), the amount of apoptotic cells was not increased in the group of 5-FU 20  $\mu$ M (apoptotic cells <1%). Much more apoptotic cells were observed in the NGF 1 mg/ml group (39%) and the 5-FU 20  $\mu$ M combined with NGF 1 mg/ml group (41%), suggesting that the apoptotic effect of the combination group is supplied mainly by NGF. 5-FU 5-fluorouracil, NGF notoginseng flower extract



with hypertension, and for acute sore throat". Modern pharmacological researches on notoginseng have found that notoginseng exerts various effects on the blood, cardio cerebral vascular system, central nervous system, endocrine system inflammation, etc. [32]. Recently, a few studies showed that the root extract of notoginseng possesses an anti-cancer effect [14, 15]. However, since the part of notoginseng commonly

used in remedies is the root, the effects of other parts of this plant have not been well studied.

The bioactive constituents of notoginseng extract are believed to be the saponins. All the notoginseng saponins are dammarane glycosides [30]. Made from different plant parts, notoginseng extracts contain a variety of saponins, and their pharmacological effects are connected with their unique chemical compositions



**Fig. 5** Anti-proliferation effects of ginsenosides Rb1, Rb3, Rc and Rg3 on HCT-116 cells assayed by MTS method. HCT-116 cells were exposed to ginsenosides for 48 h and cell proliferation was determined by MTS (mean  $\pm$  SE,  $n = 3$ ). Ginsenoside Rb1, Rb3 and Rc do not decrease the cell proliferation at 30–300  $\mu$ M. Ginsenoside Rg3 inhibits cell growth at 100 and 300  $\mu$ M significantly

[30]. Qualitative and quantitative determination of herbal medicine is an important issue for the safety and efficacy of herbal medicine [3, 29]. Some analytical studies have been conducted for the determination of the saponins of notoginseng [8, 26]. However, almost all studies have focused on the root extract. In this study, we analyzed the saponin composition of NGF. Compared with the root extract [8, 26], the chemical composition of notoginseng flower has special aspects. In notoginseng root, the main constituents are notoginsenoside R1, ginsenoside Rb1, Re and Rg1. However, for the NGF, the main constituents were ginsenoside Rb3 and Rc, while notoginsenoside R1, ginsenoside Re and Rg1 were present in very limited amounts. Ginsenoside Rg3, a previously identified anti-cancer ginsenoside [25], determined in NGF (5.2 mg/g), showed strong anti-proliferation activity on HCT-116 cells (Fig. 5). The saponin content and proportions influence the effect of notoginseng extracts. HPLC data for the determination of the saponins of NGF provide useful information and will prove advantageous for future studies.

Standardization is an important subject in herbal medicine research. It is achieved by reducing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing process. Studies of standardization of herbal extracts include many works such as the quality control on cultivation, harvest, extraction and chemical analysis. In this study, we assayed the chemical composition of NGF. Although it is not a standardization method, HPLC supplied an analytical approach for the upcoming standardization studies.

We evaluated the anti-cancer effect of extracts from the root and flower of notoginseng on colorectal cancer cells. Since the flower extract of notoginseng showed a stronger anti-proliferation effect on HCT-116 cells than that of the root extract, NGF was selected for the anti-cancer research in our current study. The time and dose dependent effects on HCT-116 cells showed that after 48 h treatment of NGF, the proliferation of cells was stable. Consequently, we selected 48 h as the treatment time. Although 5-FU has certain anti-cancer effects on colorectal cancer cells, the present study showed that even when the treatment concentration of 5-FU 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 5-FU although they lead to severe side effects [18]. Decreasing the dose of chemotherapy and increasing the anti-cancer effect by combining 5-FU with other medicines are important considerations.

In this study, the anti-proliferation effect of NGF combined with 5-FU was investigated. Compared with non-treatment control, when cells were treated with 5-FU 5  $\mu$ M or NGF 0.25 mg/ml separately, cell proliferation was reduced by 31.1 and 25.3%, respectively. The combination of 5-FU 5  $\mu$ M and NGF 0.25 mg/ml reduced cell proliferation by 59.4% (Fig. 3a). This is higher than the reduction in proliferation observed with treatment with 5-FU 100  $\mu$ M (50.5%), suggesting that combining NGF with 5-FU can reduce the dose of 5-FU needed, and significantly increasing the anti-proliferation effect on HCT-116 cells. Since it is well known that 5-FU has cytotoxic effects on primary cells, this synergistic effect between NGF and 5-FU makes it possible to reduce the dose of 5-FU in combination with NGF and thereby further decrease dose-related toxicity.

Many chemical compounds can be selected as chemotherapy for cancer treatment. Different medicines may have different effective targets on the cancer cells [24]. If two or more drugs have different effective targets on the cancer cells, combinations of these drugs may have high potency in the inhibition of cancer cell growth. 5-Fluorouracil is a widely used and well-studied cancer therapeutic medicine for the treatment of colorectal cancer. Thymidilate synthase, one of the key enzymes controlling DNA replication, is a target for several antineoplastic drugs of which 5-FU is the main one [16]. 5-Fluorouracil also influences the synthesis of RNA. 5-Fluorouracil can induce apoptosis of HCT-116 colorectal cancer cells; however, it requires very high doses and a long treatment time [13]. In this study, we use a very low dose of 5-FU to investigate its anti-pro-

liferation effects. Treatment of 5-FU 5  $\mu$ M for 48 h or 20  $\mu$ M for 8 h was not found to have an apoptotic effect (Fig. 4). After treatment with NGF 0.25 mg/ml for 48 h, apoptotic cells were observed (Fig. 4a). When the treatment concentration of NGF (1 mg/ml) was increased to an amount similar to that used of 5-FU (20  $\mu$ M), a very strong apoptotic effect was observed and the induction time of cell apoptosis was shortened (Fig. 4b). Different from the single use of 5-FU, apoptosis was observed after treatment with a combination of NGF and 5-FU. Thus, it is suggested that the apoptotic effect of the combined treatment was mainly contributed by NGF. This evidence suggested that the synergistic effect of 5-FU and NGF might come from the two drugs having different effective targets.

Dietary supplementation has been prevalent in cancer therapy for a long time. The earlier application of dietary supplements in the treatment of cancer was to prevent therapy-induced side effects [2]. The use of complementary and alternative medicine including botanical extracts is becoming increasingly popular among cancer patients. 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 [20, 31]. Using a rat model, we evaluated the preventative effect of different botanical extracts on chemotherapy-induced nausea and vomiting [27, 28]. American ginseng was found to reduce gastric side effects induced by cisplatin [17]. In this study, notoginseng, a close relative of American ginseng, was found to have a synergistic anti-proliferation effect on HCT-116 human colorectal cancer cells. In the future, we will investigate whether NGF has the ability to reduce the side effects induced by 5-FU.

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