Published in final edited form as:

J Appl Biomed. 2018 November ; 16(4): 311–319.

Red American ginseng enhances the effect of fluorouracil on human colon cancer cells via both paraptosis and apoptosis pathways

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Abstract

Introduction: As a commonly used chemotherapeutic agent, fluorouracil (5-FU) has serious dose-limiting side effects. In this study, we evaluated the synergy between red American ginseng (RAG) and 5-FU on human colorectal cancer cells, and explored the potential mechanisms.

Methods: Ginsenoside contents of white American ginseng (WAG) and RAG were determined by HPLC. Cell proliferation was evaluated by MTS assay. Combination Index (CI) analysis was executed using CompuSyn software. Paraptotic events were observed after crystal violet staining. Cell cycle distribution, cyclin A expression and apoptotic induction were analyzed using flow cytometry.

Results: We observed the heat treatment remarkably increased levels of ginsenoside Rg3, 20R-Rg3, Rk1 and Rg5. When the combinations of 5-FU and RAG were applied, cell proliferation inhibition rates were notably increased, indicating that RAG significantly enhanced 5-FU's effect. Additionally, CI analysis suggested that there was a synergistic action of 5-FU and RAG when combined. The cell cycle data indicated 5-FU induced S phase arrest, and the combination of 5-FU and RAG increased G1 phase. Further, the RAG's ability to enhance the anti-cancer effects of 5-FU was linked to both paraptosis and apoptosis inductions.

Conclusion: RAG may have clinical utility to decrease the dosage of 5-FU in colorectal cancer therapeutics.

Keywords

Red American ginseng; Fluorouracil; colorectal cancer; Cell cycle; Paraptosis; Apoptosis

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Conflict of interests

No conflict of financial or other interests exists in the submission of this manuscript. The work was an original research which has not been published previously, and not under consideration for publication elsewhere, in whole or in part. The content of this manuscript is approved by each listed author for publication.

Introduction

Human colorectal cancer is a significant public health issue worldwide (Rajpal and Venook, 2006; Siegel et al., 2016). Although the early stages of this malignancy can be cured by surgical resection alone, most often a combination of surgery and adjuvant chemotherapy and radiotherapy is the choice of treatment. Chemotherapies are often limited by their severe side effects and by dose-limiting toxicity. The drug-related adverse events not only worsen patients' quality of life, but can also lead to patient noncompliance in continuing the potentially curative chemotherapy (Schnell, 2003; Smorenburg et al., 2006).

Fluorouracil (5-FU) is a commonly used chemotherapeutic agent that interferes with the growth of cancer cells, including metastatic colorectal cancer cell (Pinedo and Peters, 1988). However, this drug has serious side effects including nausea, fatigue, and a decrease in blood cell counts (Delval and Klastersky, 2002). In the treatment of advanced cancer, higher 5-FU doses are not more effective yet increase side effects in both clinical and laboratory studies (Meregalli et al., 1998). For experimental cancer research, among several human colorectal cancer cell lines, HCT-116 is a very commonly used cell model (Mohr and Illmer, 2005; Wang et al., 2012).

Many effective anti-cancer drugs have been developed from botanical sources (Cai et al., 2015; Li and Zhang, 2008; Pang et al., 2015). Patients with cancer also often use herbal medicines to decrease the side effects of chemotherapy and to improve their well-being (Barton et al., 2013; Ott, 2002). Attempts have been made to identify effective herbal medicines in cancer therapeutics (Han et al., 2015; Zhang et al., 2015). Clearly, there is a need to enhance 5-FU's anti-cancer activity and to reduce its toxicity in patients with colorectal cancer. Thus, new medications from potential herbal sources should be explored.

Ginseng root has been used for centuries in Oriental medicine as a panacea that promotes longevity (Attele et al., 1999; Qi et al., 2011b). Previous ginseng investigations, including anti-cancer research, have demonstrated the beneficial effects of both Asian ginseng and American ginseng (Qi et al., 2011a; Wang et al., 2015a). We have reported that untreated ginseng can augment the pharmacological effects of 5-FU (Li et al., 2009; Wang et al., 2007b). Like Asian ginseng, white American ginseng (WAG) is prepared by air-drying. If fresh American ginseng is processed by steaming, from white color to red, the steamed product is called red American ginseng (RAG). During the steaming process, extensive conversion of original ginsenosides in white ginseng to new degradation compounds in red ginseng was observed, leading to different ginsenoside profiles (Sun et al., 2011). Since American ginseng has significantly better anti-colorectal cancer activity after heat-steaming treatments (Wang et al., 2007a), it is possible that RAG possesses a better ability to enhance the action of 5-FU. In this study, we investigated the anti-proliferative effects of RAG and/or 5-FU on HCT-116 human colorectal cancer cells. Specifically, the effects on the cancer cell cycle and cyclin A were analyzed, and the mechanisms of action in relation to paraptosis and apoptosis were explored.

Materials and methods

Chemicals and materials

Ginsenoside standards used in this study, purchased from Delta Information Center for Natural Organic Compounds (Xuancheng, AH, China), were of biochemical-reagent grade and were at least 98% pure, as confirmed by HPLC. 5-FU was obtained from American Pharmaceutical Partners Inc. (Schaumburg, IL, USA). HPLC grade ethanol, *n*-butanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q water was supplied by a water purification system (US Filter, Palm Desert, CA, USA). Trypsin, McCoy's 5A medium, fetal bovine serum (FBS), penicillin/streptomycin solution (200×), propidium iodide (PI) and RNase were obtained from Mediatech, Inc. (Herndon, VA, USA). A CellTiter 96 Aqueous One Solution cell proliferation assay kit was obtained from Promega (Madison, WI, USA). Annexin V- fluorescein isothiocyanate (FITC) and cyclin A-FITC were obtained from BD Biosciences (San Diego, CA, USA).

Plant material and extract preparation

The root of American ginseng (*P. quinquefolius*) was obtained from Roland Ginseng LLC (Wausau, WI, USA). The roots were air-dried to produce WAG, while the roots were steamed at 120 °C for 2 h to produce RAG. WAG and RAG roots were further lyophilized to obtain dried samples for the following extraction. These dried roots were ground and extracted with 70% ethanol. The solvent of the extract solution was evaporated, and then re-dissolved in water. The aqueous solution was extracted with water-saturated *n*-butanol. The *n*-butanol phase was evaporated under vacuum and then lyophilized to obtain WAG and RAG extracts, respectively.

HPLC analysis

HPLC analysis was conducted on a Waters 2960 instrument with a Waters 996 photodiode array detector (Milford, MA, USA). The separation was carried out on an Alltech Ultrasphere C18 column (5 μ m, 250 $\times$ 3.2 mm I.D.) (Deerfield, IL, USA) with a guard column (Alltech Ultrasphere C18, 5 μ m, 7.5 $\times$ 3.2 mm I.D.). 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; to 26% A for 3 min and was held for 19 min; to 36% A for 13 min; to 50% A for 9 min; to 95% A for 2 min and was held for 3 min and finally was changed to 18% A for 3 min and held for 8 min. The flow rate was 1.0 ml/min and the detection wavelength was set to 202 nm. All the tested solutions were filtered through Millex 0.2 μ m nylon membrane syringe filters (Millipore Co., Bedford, MA, USA) before use. The levels of ginsenosides in each sample were calculated using standard curves of ginsenosides.

Cell culture

HCT-116 human colorectal cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with McCoy's 5A containing 10% PBS, 50 IU penicillin/streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C.

Cell proliferation analysis

WAG and RAG extracts were dissolved in 50% ethanol and were stored at 4 °C before use. 50 mg/ml of 5-FU was diluted with milli-Q water. Cells were collected and separated into single-cell suspensions with McCoy's 5A medium, and then were seeded in 96-well plates with the density of 1×10^4 cells/well. After 24 h, culture medium was removed before various concentrations of American ginseng extracts and 5-FU were added either individually or combined to the wells. The final concentration of ethanol in the ginseng group was 0.5%. Controls were exposed to culture medium containing the same quantity of ethanol without drugs. After treatment for 48 h, cell growth was evaluated using an MTS assay according to the manufacturer's instructions. The medium was replaced with MTS solution (20 μ l of MTS reagent diluted in 100 μ l of fresh medium) before being returned to the incubator for another 2 h at 37 °C with 5% CO₂. A 60 μ l aliquot of medium from each well was transferred to an ELISA 96-well plate and its absorbance at 490 nm was recorded. Each experiment was performed in triplicate.

Combination index analysis

The synergism studies of 5-FU and RAG were carried out using CompuSyn software 1.0 (Combosyn Inc., Paramus, NJ, USA). Combination index (CI) was recognized as the standard measure of drug combination effect based on Chou–Talalay equation $CI = (d_A/D_A) + (d_B/D_B)$. In which, D_A and D_B represents the dose of drug 1 and drug 2 in the combination required to achieve the same efficacy as those of drug 1 (d_A) and drug 2 (d_B) when used alone (Chou, 2006). The result CI>1, CI = 1 and CI<1 indicate antagonistic, additive and synergistic effect, respectively. Dose-effect curve, Fa-CI plot and normalized isobologram were generated from CompuSyn.

Cell cycle and cyclin A analysis

Cells were seeded in 24-well plates (1×10^5 cells/well). The medium was replaced 24 h after seeding with fresh medium containing 5-FU (30 μ M), RAG (100 μ g/ml), or both. To analyze the cell cycle distribution, cells were trypsinized after 48 h of exposure to these drugs, fixed gently with 80% ethanol in a freezer for 2 h and were treated with 0.25% Triton X-100 for 5 min in an ice bath. The Cells were resuspended in 300 μ l of PBS containing 40 μ g/ml propidium iodide (PI) and 0.1 mg/ml RNase. Then 20 μ l of cyclin A-FITC was added to the cell suspension. Cells were incubated in a dark room for 20 min at room temperature and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, at least 20,000 cells were counted.

Crystal violet staining assay

The cells were seeded in 24-well plates (1×10^5 cells/well). After 24 h, the medium was replaced and various concentrations of RAG and/or 5-FU were added to the wells. After 48 h of drug exposure or control conditions, the medium was removed and the cells were washed and stained with 0.2% crystal violet in 10% phosphate-buffered formaldehyde for 2 min. The staining solution was removed and the cells were washed twice with PBS. The

remaining cells adhering to the wells were observed under the microscope and photographed.

Apoptosis analysis

Cells were seeded in 24-well tissue culture plates (1×10^5). After 24 h, the medium was replaced and 5-FU (30 μ M), RAG (100 μ g/ml), and RAG (100 μ g/ml) + 5-FU (30 μ M) were added. After treatment for 24 or 48 h, floating cells in the medium and adherent cells were collected. 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. For each measurement, at least 20,000 cells were counted.

Statistical analysis

Data were presented as the mean $\pm$ standard error (SE). A one-way ANOVA was employed to determine whether the results had statistical significance. In some cases, a Student's *t*-test was used to compare two groups. The level of statistical significance was set at $p < 0.05$.

Results

Ginsenosides HPLC analysis

Fig. 1 shows the ginsenoside content differences between WAG and RAG. After the steaming treatment, there were remarkable conversions of individual ginsenosides. With level reductions in ginsenosides Rg1, Re, Rb1, Rc and Rd, the levels of ginsenosides Rh1, Rg2, 20*R*-Rg2, Rg3, 20*R*-Rg3, Rk1 and Rg5 were obviously increased.

Anti-proliferative effects of 5-FU and ginseng extracts on HCT-116 cancer cells

The effects of WAG and RAG on the proliferation of HCT-116 cell line were tested using a MTS assay. As shown in Fig. 2A, exerted RAG showed an effect at 30 μ g/mL (7.4%) after 48 h treatment, while WAG did not have obvious cytotoxic effects up to 150 μ g/ml. Different concentrations of RAG were selected based on the initial experiments to investigate the synergistic potential on 5-FU-induced growth inhibition of HCT-116 cells (Fig. 2B). Results of cell viability revealed that RAG significantly enhanced the anti-proliferative effect of 5-FU in a dose-dependent manner (Fig. 2C). Although 5 μ M 5-FU alone did not have an obvious effect (5.1%), the combination of 5-FU with 30 μ g/ml RAG significantly inhibited the cancer cell growth (18.2%; $p < 0.01$). When 30 μ M 5-FU and 100 μ g/ml RAG were used alone, the cell proliferation inhibition rates were at 25.2% and 24.6%, respectively. When they were used together, cell proliferation was inhibited by 83.5% ($p < 0.01$).

We utilized CompuSyn software to evaluate the combined effect of RAG and 5-FU in HCT-116 cells. As shown in Fig. 2D, the CI values for RAG plus 5-FU in different combinations were calculated and plotted with the percent of cell growth inhibition as the fraction affected (Fa) cells. The symbols of all the combinations were found to be below the CI = 1 horizontal line, signifying anti-proliferative synergism of 30/100 μ g/mL RAG plus 5/10/30 μ M 5-FU in HCT-116 cells. 100 μ g/mL RAG and 30 μ M 5-FU co-treatment exhibited strong synergism as it generated a lower CI value (0.23). Normalized isobologram

was presented in Fig. 2E, which helps to verify the synergistic action of RAG and 5-FU in combination treatment.

Effects of RAG on 5-FU-induced changes in cell cycle and cyclin A

We examined whether the inhibition of the cancer cell growth is a consequence of the cell cycle being arrested at a specific phase. As shown in Fig. 3, the cell cycle profile in the control group was 56.6% G1, 24.9% S and 18.5% G2/M. After treatment with 30 μ M 5-FU, the cell cycle profile was changed to 11.0% G1, 81.4% S and 7.6% G2/M, indicating 5-FU significantly induced the S phase arrest of the cell cycle. After treatment with 100 μ g/ml of RAG only, the cell cycle arrest in G1 phase increased to 63.1%. Compared with the percentage of cells at the G1 phase of the cycle after treatment with 5-FU only (11%), treated with the combination of these two compounds together, the percentage of cancer cells in G1 phase was increased to 30.1% ($p < 0.01$), while the percentage of cells in the S phase was reduced (Fig. 3C). The S phase data was supported by the expression of cyclin A (Fig. 3D), in which it is linked primarily to S phase (Lim et al., 2007).

Effects of RAG and 5-FU on induction of paraptosis-like cell death

To explore the potential mechanism of the ginseng-induced cell death, morphological observation was performed after crystal violet staining since visible cytoplasmic vacuolization is a typical feature of paraptosis (Chen et al., 2008). As shown in Fig. 4A, after treatment with the ginseng, cytoplasmic vacuolization was obviously present in the cancer cells, while the cells treated with the vehicle control and 5-FU did not show any vacuoles. When the concentration of the two test compounds increased, the vacuole induction rate increased accordingly (Fig. 4B). A RAG concentration of 100 μ g/ml had a vacuole induction rate of 34.6%, and this paraptosis-like cell death was significantly enhanced when in combination with 5-FU at 10 μ M (48.4%) and 30 μ M (74.2%) ($p < 0.05$ and $p < 0.01$, respectively).

Effects of RAG on 5-FU-induced cancer cell apoptosis

Fig. 5 shows that HCT-116 cancer cells were treated with 100 μ g/ml RAG in the absence or presence of 30 μ M 5-FU. Apoptosis was quantified by flow cytometric analysis. At 24 h and 48 h, 5-FU and RAG alone induced cancer cell apoptosis by 6.4% vs. 5.8% and 8.2% vs. 9.4%, respectively. However, at 24 and 48 h, the combination of the two compounds very significantly increased the rate of cell apoptosis induction by 23.8% and 45.5%, respectively.

Discussion

5-FU is one of the most widely used chemotherapeutic agents in colorectal cancer treatment (Pinedo and Peters, 1988; Smorenburg et al., 2006). For the treatment of metastatic colon cancer, however, higher 5-FU doses produce more adverse events, and may not be more effective than lower doses (Meregalli et al., 1998). To improve the treatment of advanced cancer, methods of improving 5-FU's efficiency are needed. Combining 5-FU with botanicals can decrease the intended 5-FU dose, while increasing the drug's anti-cancer effect, and the patient may benefit from the combination treatment regimen.

In this study, using a HCT-116 colorectal cancer cell proliferation test, we observed that RAG significantly enhanced 5-FU's anti-proliferative effects. Drug synergism was studied using CompuSyn software, which illustrated the synergistic interaction of RAG and 5-FU as indicated by a CI value of the combination dose that was less than 1 ($CI < 1$). The cell cycle analysis showed that while 5-FU significantly induced the S cell cycle phase arrest, red ginseng obviously induced G1 phase cycle arrest. Co-treatment with the two compounds significantly increased the cells at the G1 phase, compared to 5-FU alone. The different effects 5-FU and RAG on cell cycle arrest likely provide a pharmacological basis for the observed synergistic effect on HCT-116 cancer cells.

Paraptosis, featured with cytoplasmic vacuolation, is a type of programmed cell death morphologically distinct from apoptosis and necrosis (Chen et al., 2008; Wang et al., 2013). After 5-FU and RAG co-treatment, accumulation of cytoplasmic vacuoles was remarkably increased in the cancer cells, indicating that paraptosis was significantly increased. Consistent with previous reports (Choudhary et al., 2012), we observed that 5-FU induced some cell death, but not to an impressive extent. Similar effects on the induction of apoptosis were observed when RAG was administered alone. However, after co-administration of these two compounds, our data showed that the induction of apoptosis increased very significantly, further supporting the synergistic effect of 5-FU and ginseng. Apoptosis is considered to be an important mechanism in the inhibition of cancer cells, and many cancer chemotherapeutic agents are strong inducers of apoptotic against cancer cells. Our data suggest that both paraptosis and apoptosis pathways are involved in the reported anti-colorectal cancer effect.

In the reported botanical anti-cancer studies, herbal medicines have been used alone or in combination with oncology drugs (Wang et al., 2015b; Yang et al., 2015; Zhu et al., 2015). While identifying combinatory therapies using natural product-derived compounds to increase cancer drug effectiveness has great potential, undesirable herbal-drug interactions should be evaluated. Previous pharmacokinetic observations in rats have shown that American ginseng does not affect 5-FU plasma levels (He et al., 2015), suggesting no pharmacokinetic interactions exist between these two compounds.

The saponin content and proportions in ginseng influence its pharmacological effects. Our HPLC data for the determination of the ginseng saponins provided useful information. Standardization is an important subject in herbal medicine research that aims to minimize the inherent variation in natural product composition through quality assurance practices applied to the agricultural and manufacturing process (Yu et al., 2014). Studies of the standardization of herbal extracts include many efforts such as the quality control of cultivation, harvest, extraction and chemical analysis. In this study, we assayed the chemical composition of the red ginseng extract. Although a standardization method has not been established for red ginseng, HPLC is an analytical approach which can be applied to the future quality control of botanicals.

The enteric microbiome plays an important role in converting ginseng parent compounds to their metabolites after oral American ginseng administration (Sekirov et al., 2010; Tawab et al., 2003). Ginseng metabolites likely possess much better anti-cancer activities than their

parent compounds (Gao et al., 2013; Wang et al., 2012). We previously demonstrated that the human enteric microbiome converts ginseng saponins into metabolites, and we detected over 20 metabolites (Wan et al., 2013). In this study, we used a heat-steaming process to prepare RAG, to mimic the ginsenoside chemical structural conversions by the enteric microbiome. As shown in Fig. 6, for ginsenosides in both protopanaxadiol and protopanaxatriol groups, ginseng steaming generated new ginsenoside derivatives, such as ginsenosides Rg3, 20*R*-Rg3, Rg5, Rk1 and Rh1. These compounds and other derivatives are likely responsible for the increases in anti-cancer activities compared to their parent compounds (Wang et al., 2012; 2013).

Conclusion

In summary, this study evaluated the effects of RAG and/or 5-FU on HCT-116 human colorectal cancer cells. Co-administration of RAG and 5-FU significantly inhibited cell growth. Importantly, this effect could not be achieved when untreated ginseng was used. The co-treatment showed a greater increase in the cell count at the G1 phase than did 5-FU alone, and this difference in cell cycle arrest contributed to the observed synergistic effect. The ability of RAG to enhance the anti-cancer effects of 5-FU was likely linked to both paraptosis and apoptosis inductions. As a compound derived from a natural product, RAG may have clinical value in the management of colorectal cancer.

Acknowledgements

This work was supported in part by grants from the NIH/NCCAM AT004418 and AT005362, the National Natural Science Foundation of China (81603378), the Natural Science Foundation of Jiangsu Province (BK20160545), China Postdoctoral Science Foundation (2017M610830).

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Fig. 1. HPLC analysis of major ginsenosides in white and red American ginseng root extracts. (A) Representative chromatograms of white American ginseng (WAG, upper panel) and red American ginseng (RAG, lower panel). American ginseng root was steamed at 120 °C for 2 h. (B) Contents of ginsenosides in the WAG, and RAG extracts. Rk1 and Rg5 are not included. Note the scale difference in the content (%).

Fig. 2.

RAG in combination with 5-FU inhibited the growth of HCT-116 human colorectal cancer cells after 48 h treatment. (A) Anti-proliferative effects of WAG and RAG on HCT-116 cells measured by MTS assay. (B) Effect of treatment with 5-FU alone or in combination with RAG on HCT-116 cell proliferation. Data obtained from triplicate experiments. (C) Dose-effect curve. (D) The CI values for RAG and 5-FU in different combinations calculated by Chou–Talalay method using CompuSyn software, and plotted with the percent of cell growth inhibition as the fraction affected (Fa) cells. The symbols below the CI = 1 horizontal line indicate synergism, above the line indicate antagonism, or on the line indicate additive effect. (E) Normalized isobologram for the combination of RAG and 5-FU. The combination data point on the lower-left of the hypotenuse is synergistic, on the upper-right of hypotenuse is antagonistic or on the hypotenuse is additive.

Fig. 3.

Effects of RAG on 5-FU-induced cell cycle arrest and the expression of cyclin A. HCT-116 cancer cells were treated with RAG at 100 $\mu\text{g/ml}$ in the absence or presence of 5-FU (30 μM) for 48 h. The cell cycle and cyclin A were assessed with PI/RNase and cyclin A-FITC staining using flow cytometry. (A) The representative histograms of DNA content in different experimental groups. (B) The expression of cyclin A. The numbers are a percentage of cyclin A positive cells. (C) Percentage of each cell cycle phase in different groups. (D) Percentage of cyclin A-positive cells in different groups. Data obtained from triplicate experiments. * $p < 0.05$; ** $p < 0.01$ compared to control. # $p < 0.05$; ## $p < 0.01$ compared to 5-FU alone.

Fig. 4.

Induction of RAG and 5-FU on paraptosis-like cell death. (A) HCT-116 cancer cells were treated with 30–100 $\mu\text{g/ml}$ RAG with or without 10–30 μM 5-FU for 48 h, and were then stained with crystal purple and photographed. (B) The percentage of total cell numbers with cytoplasmic vacuoles. Data obtained from triplicate experiments. # $p < 0.05$; ## $p < 0.01$ vs. RAG alone.

Fig. 5.

Effects of RAG on 5-FU-induced apoptosis. HCT-116 cancer cells were treated with 100 $\mu\text{g/ml}$ RAG in the absence or presence of 30 μM 5-FU for 24 and 48 h. Apoptosis was quantified using annexin V/PI staining followed by flow cytometric analysis. (A) Representative scatter plots of PI (y-axis) vs. Annexin V (x-axis) in each experimental group. 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); late apoptotic or necrotic 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). (B) Percentage of apoptotic cells. Data obtained from triplicate experiments. * $p < 0.05$; ** $p < 0.01$ compared to control. ## $p < 0.01$ compared to 5-FU alone.

Fig. 6.

Chemical structural changes during the steaming process. For the protopanaxadiol group ginsenosides, Rb1, Rc and Rd were converted to Rg3 and 20R-Rg3, then to Rg5 and Rk1. For protopanaxatriol group ginsenosides, Re is converted to Rg2 and 20R-Rg2; Rg1 is further converted to Rh1. Steaming-generated ginsenoside derivatives have a significantly stronger anti-cancer potential than their parent compounds.