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Original Article

Apoptosis Induction with Enhancement of BAX/BCL2 Gene Expression Ratio via Combination Therapy in HT29 Colon Cancer Cells

Roya Bazzaz[1], Parichehreh Yaghmaei[1], Siavoush Dastmalchi[2], Nadereh Rashtchizadeh[3, 4*]

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2. Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
3. Connective Tissue Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
4. Department of Clinical Biochemistry, Sarab University of Medical Sciences, Sarab, Iran.

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Abstract

Background and Aim: Combination therapy is one of the new strategies that minimize resistance to chemotherapy and reduces drug toxicity. Here, we investigated the effect of combination therapy with 5-Fluorouracil and Gamma Tocopherol on cell survival and BAX/BCL2 gene expression ratio in HT29 colon cancer cells.
Methods: The proliferation of cancer cells was determined via colony formation assay.
BAX/BCL2 ratio was evaluated after incubation with concentrations of 5-Fluorouracil and Gamma Tocopherol via real-time-PCR.
Results: The average number of colonies in the cells treated with 5-Fluorouracil, Gamma Tocopherol and their combination of them was 63±4, 78±3, and 28±2, respectively which significantly decreased in the combination group. In contrast with the control group, the BAX/BCL2 ratio remarkably increased when the cell underwent combinational treatment ($p<0.05$).
Conclusion: 5-Fluorouracil and Gamma Tocopherol reduced HT 29 cell proliferation. Our results suggest that combination therapy with 5- Fluorouracil and Gamma Tocopherol can be considered as a strategy for induction of apoptosis via increasing the BAX/BCL2 ratio.

Keywords: Colon cancer; Apoptosis; 5-fluorouracil; Combination Therapy; γ-tocopherol.

***Corresponding Author:** Nadereh Rashtchizadeh; **Email:** rashtchizadeh@rocketmail.com

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Introduction

It is well-established that cancer is the second cause of death following heart disease. Moreover, it is the third cause of death following accidents and heart disease in Iran (1). Specifically, colon cancer is the third most common cancer in the world and is the second and fourth most common cancer in Iran (2). Previous studies have demonstrated that the most important risk factors of colorectal cancer include aging, colorectal polyps, familial history of colon cancer, genetic disorders, inflammatory bowel disease, unsuitable diet, and smoking (3). Under normal conditions, there is a balance between programmed cell death (apoptosis) and cell viability, and disorders of these mechanisms can lead to cancer progression (4). Some drugs and materials are effective on the cell cycle and affect different pathways of these cell events. Among these agents, gamma-tocopherol (γT) as one of the eight isomers of vitamin E, has strong anti-cancer properties (5). This molecule is considered an effective anti-oxidant agent since it reduces reactive oxygen and nitrogen species, increases glutathione, and induces enzymes that break down and remove free radicals (6). Other anti-cancer properties of this

molecule include inhibition of lipoxygenase and cyclooxygenase enzymes, reduction of the isoprostane (one of the isomers of prostaglandin F2), and inhibition of tumor necrosis factor (TNFα) as an inflammatory agent, all of which mitigate inflammation and oxidative stress (7). Programmed cell death (PCD) occurs via external and internal (mitochondrial) pathways and is organized by BCL2 family genes (8). This family includes the anti-apoptotic genes B-cell lymphoma 2 (BCL2), B-cell lymphoma-extra-large (BCL-X (L)), Myeloid leukemia cell differentiation protein (MCL1), as well as the pro-apoptotic genes BCL2 associated agonist of cell death (BAD), Bcl-2 homologous antagonist/killer (Bak) and Bcl-2-associated X protein (BAX) (9). The regulation of expression of these pro and anti-apoptotic proteins leads to the activity of apoptotic pathways; on the other hand, the pro-apoptotic and anti-apoptotic genes expression ratio determines the cell survival or apoptosis. Thus, there is a positive correlation between apoptosis and the effectiveness of chemotherapy (10). Gamma tocopherol induces expression of BAD, BAX, P21 (a cyclin-dependent kinase inhibitor), P53 (a tumor suppressor protein), and caspase 3 genes by increasing PPARα expression. Also, by reducing the mRNA level of the anti-apoptotic BCL2 gene, leads to cell death and stops the cell cycle by inhibiting cyclin-dependent kinase (CDK) (11).

According to the previous studies on different cell lines, BCL2 is known as an inhibitory factor of apoptosis and BAX is a key factor in the induction of cell death (12). In colon cancer, increasing BCL2 expression decreases the BAX/BCL2 gene expression ratio leads to impaired apoptosis, as well as resistance to anticancer drugs and radiotherapy (13). This ratio within the cell regulates the cell's ability to respond to the apoptotic signal. In other words, the cells with a higher level of this ratio are more sensitive to apoptotic stimulators than cells with a lower ratio (14). In colon cancer treatment protocol, several drugs are used during chemotherapy and induce apoptosis. One of these drugs is 5-fluorouracil (5-FU), which is an analog of uracil and belongs to the antimetabolites family (15). This drug has severe side effects (16) and finding new treatment strategies can reduce its detrimental effects thereby increasing increase patient satisfaction. Combination therapy is one of the new strategies that minimize resistance to chemotherapy and reduces drug toxicity (17).

In this study, we used gamma-tocopherol as an adjuvant in addition to the 5-fluorouracil to enhance the drug efficacy in stimulating apoptosis. For the first time, this work shows that the use of 5FU and gamma-tocopherol in combination therapy reduced cell proliferation and modified the BAX/BCL2 ratio in the Human Colorectal Adenocarcinoma Cell Line (HT29).

Methods

5-fluorouracil (5-FU), γ-tocopherol, Roswell Park Memorial Institute (RPMI) 1640 medium, streptomycin, glutamine, penicillin G, and RNA extraction kit (TRI Reagent) were purchased from Sigma-Aldrich (Germany). The human colon cancer cell line (HT-29) was procured from the National Cell Bank (Pasteur Institute, Iran). Fetal bovine serum (FBS) and PBS (Phosphate Buffered Saline) were obtained from GiBCo Invitrogen (USA). PCR Master Mix was purchased from Jena bioscience (Germany) and complementary DNA (cDNA) synthesis kit from Thermo Science (USA). The HT-29 colon cancer cells were cultured in RPMI medium supplemented with 100 U/mL penicillin, 10% FBS, 100 mg/mL streptomycin, at 37 °C incubators with 5% CO2. Cells were detached from the flask with 0.5 mL trypsin–EDTA (Ethylene Diamine Tetra Acetic acid) solution and passaged. Drug stocks (100 mM) were made with dissolving 5-FU and γ-tocopherol in Dimethyl sulfoxide (DMSO) and different concentrations of drugs (5,10 μM) were prepared via stock dilution in RPMI 1640 medium (18).

Cell Proliferation assay

To assess proliferation and cell survival, the colony formation assay was carried out. It is essential to measure the proliferative capacity of treated cells to provide a measurement of cell death. This can be achieved using the colony-forming assay (CFA). Single tumorigenic cell with a high proliferation rate forms colonies in a few days. CFA monitors a

cancer cell's ability to produce a viable colony after treatment.

Colon cancer cells were cultured in RPMI-1640 medium and counted using a hemocytometer. These cells were seeded into a six-well culture plate containing RPMI 1640 medium (5×10^2 cells/well) and incubated at 37°C in 5% CO2. After 24 hours the cells were treated with different concentrations of drugs (10 µM 5-FU and 5 µM γ-tocopherol) in a single and combination pattern. One day later, the single-cell suspension was prepared by trypsinization. The cells were counted with a hemocytometer and incubated for additional seven days at 37° C. Then the medium was removed from each of the wells by aspiration. PBS was used for rinsing the cells. The colony fixation was carried out using sufficient 100% methanol to cover the cells for 15 minutes at room temperature. The cells were stained with sufficient crystal violet staining solution (0.01% (w/v)) to cover the cells for 5 minutes at room temperature. Finally, an excess amount of fixator and dye was washed with dH2O and allowed the plate to dry. The colonies consisting of more than 50 cells were counted using an inverted microscope (19). The cells were evaluated morphologically and digital images of the colonies were analyzed using Image J software (Version 1.14).

Quantitative real-time PCR analysis

To evaluate of BAX/BCL2 ratio, the TRI Reagent kit and Revert Aid First Strand cDNA Synthesis Kit were used for RNA extraction and cDNA synthesis respectively, according to the manufacturer's manual guide. Real-time Polymerase chain reaction (PCR) analysis was carried out using PCR Master Mix and β-actin as a reference gene (18). The reverse and forward primers sequences have been listed in table 1. Our results were evaluated as mean±standard deviation of three independent experiments. One-way ANOVA and t-test were performed to interpret the differences between control and treated groups. $p< 0.05$ was considered significant statistically. The data were analyzed with GraphPad Prism software (version 6.07).

Table 1. Primers used for quantitative real-time PCR

Target Gene	Primer sequence
BCL2 (Gene ID: 596)	Forward: 5′-CTTTAGAGTTGCTTTACGTTG-3′ Reverse: 5′-TCCATATTCATCACTTTGACAA-3′
BAX (Gene ID: 581)	Forward: 5′-TTCATGGACGGGTCCGGGGAGC-3 Reverse: 5′-TATCAGCCCATCTTCTTCCAGATGGT-3′
β-actin (Gene ID: 60)	Forward: 5'-CCTTCCTGGGCATGGAGTCCTG-3' Reverse: 5'-GAAGATCAAGATCATTGCTCC-3'

Results

Determination of the inhibitory effect of 5-FU and γ-tocopherol on cancer cell death

Macroscopic evaluation of colon cancer HT29 cells in single and combination-treated groups using crystal violet staining confirmed that 5-FU and γ-tocopherol decreased the colony formation rate in cells (figure 1). As shown in table 2, it was demonstrated that 10 µM 5-FU and 5 µM γ-tocopherol inhibited the number of colonies compared with the control group (500 cells/well and DMSO < 1%) (Table 2).

This inhibition was more effective in cells treated with a combination of drugs. In other words, in the combination group, 10 µM 5-FU and 5 µM γ-tocopherol, reduced the colony formation significantly ($p < 0.05$) (figure 2).

Evaluation of 5-FU and γ-tocopherol combination therapy on BAX/BCL2 gene expression ratio

To understand the role of 5-FU and γ-tocopherol on BAX/BCL2 ratio in cancer cells, RT-PCR was performed. 10 µM 5FU and 5 µM γ-tocopherol was used and the results were compared with untreated cells (500 cells/well and DMSO < 1%). Applying 5-FU and γ-tocopherol caused the dramatic enhancement of BAX/BCL2 ratio compared with the control group ($p < 0.05$). The most increasing effect belonged to the cells treated with a

combination of 5-FU and γ-tocopherol ($p < 0.01$) (Figure 3).

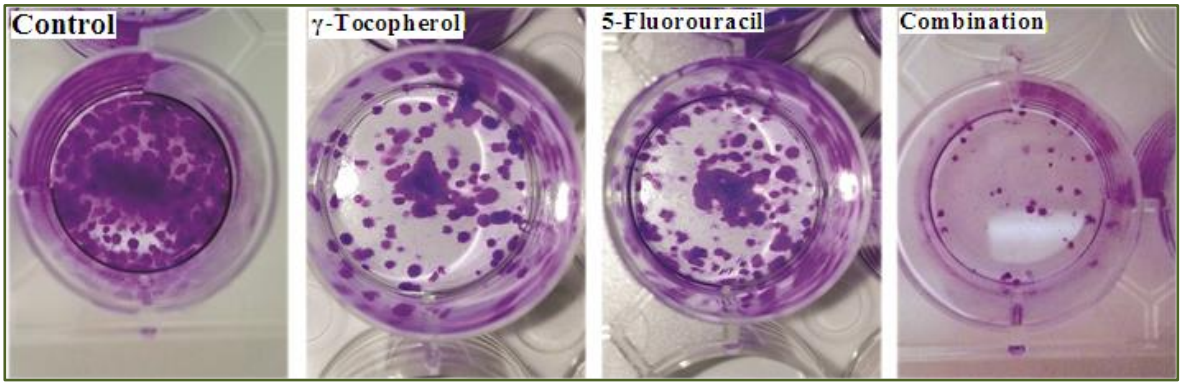

Figure 1. Colony-formation assay of HT29 cell line treated with 5-fluorouracil and γ-tocopherol. Evaluation of cell survival in single and/or combination-treated groups carried out using colony formation assay. Macroscopic pictures of treated cells confirmed that 5-FU and γ-tocopherol decreased the colony formation rate in HT29 cells, especially in the combination group.

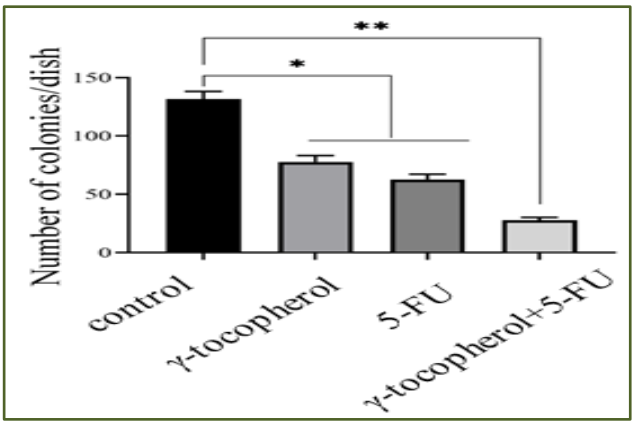

Figure 2. Effect of 5-FU and γ-tocopherol on the colony formation in HT29 cell line - 10 µM 5-FU and 5 µM γ-tocopherol decreased the number of colonies in treated cells ($p<0.05$) . Combination of drugs was more effective compared with single treatment ($p<0.01$). (n = 3). *p< 0.05, **p < 0.01.

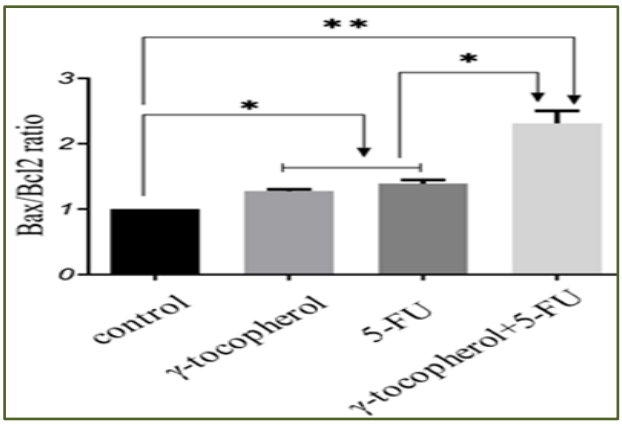

Figure 3. Effect of 5-FU and γ-tocopherol on BAX/BCL2 ratio in HT29 cell line – Real-time PCR was performed to understand the role of 10 µM 5-FU and 5 µM γ-tocopherol on this ratio. Findings showed that combination therapy caused BAX/BCL2 enhancement. The results were considered as the mean ± standard deviation (n = 3), *p< 0.05, **p< 0.01.

Table 2. The mean number of colonies formed in the medium via treated cells with 5-FU and γ-T

Treatment	Concentration	Mean colony number
Control	No drug	95 ± 5
5-fluorouracil *	Mµ10	63 ± 4
γ-tocopherol *	Mµ5	78 ± 3
5-FU + γ-tocopherol ** (Combination)	M (γT)µ5) + FU 5 (Mµ 10	28 ± 2

The number of colonies was decreased in H29 cells treated with FU and γ-tocopherol and this effect was significant in the combination group. The results represent SD ± mean. (*p < 0.05 , ** p < 0.01)

Discussion

Cancer is a disease related to unusual cell growth with potency to spread to the other parts of the body (20). The invasive, difficult, costly, and non-specific treatments, along with the growth of the number of patients with this disease have made cancer a major concern worldwide. One of the most common malignancies is colon cancer, which has a

high incidence rate in Iran due to lifestyle and cultural changes (21).

5-Fluorouracil is one of the first-line treatments of colorectal cancer (22). Our findings revealed that this drug leads to cell death by reducing the expression of the BCL2 gene. Similar to our results, Yuwei et al. concluded that in colon cancer cell lines, treatment with 5FU (IC_{50}:10.7-12.28 µM) would reduce the percentage of living cells dramatically (23-25). According to the colony formation test results, 5-FU reduced colony formation rate in HT29 cells, which was more effective in the combination of 5-FU and gamma-tocopherol (Figure1). Consistent with these findings, Ofonime et al. showed that 0.1 µM 5-fluorouracil decreased the colony formation of HT29 cells dose-dependently. Thus, a 100 µM concentration of 5-FU eliminated the possibility of colony formation (26). In cancer cells, due to low tumor suppression factors, high expression of oncogenes, and high cell cycle speed, the ROS concentration is higher, and subsequently, autophagy and metastasis occur. All of these events lead to drug resistance (27). Several strategies have emerged to inhibit this phenomenon. One of the solutions is targeted therapy which prevents a wide range of side effects by directing the drug to the desired tumor tissue. Sabzichi et al. showed that the application of chemotherapy-based nanoparticles is a powerful method of drug delivery and enhances the effectiveness of drugs in the MCF7 breast cancer cell lines while also reducing its unpleasant side effects (28). Another most widely and effective strategy is combination therapy. The advantages attributed to combination chemotherapy include increasing patient satisfaction by reducing the frequency of drug administration, synergistic effects of agents, decreasing multidrug resistance, lowering the drug dose, and consequently reducing toxicity in healthy tissues (22). Banu & et al showed that combination therapy reduced the resistance to chemotherapy drugs and provided the opportunity to trigger multiple target routes simultaneously (24). Cancerous tissues have very complex characteristics and even one part of the tumor may have different behavior from the other part of the tumor in terms of response to treatment. In these cases, combination therapy leads to better results and prevents disease progression (29).

In this study, we applied gamma-tocopherol as an adjuvant to boost the effectiveness of the main drug in the treatment of cancer cells and reduce its side effects. Similar to the results of our combined treatment study, Fu et al showed that 5-FU and Salinomycin had synergistic effects in liver cancer cell lines (17). Satari et al. also showed that Rutin along with 5 fluorouracil enhanced the effectiveness of 5-FU (26). We concluded that gamma-tocopherol enhances the inhibiting effects of 5-FU on the growth and proliferation of HT29 colon cancer cells. This isomer of vitamin E induces apoptosis by increasing dihydrosphingosine and dihydroceramide levels thereby increasing the activity of caspase 3 and 9 enzymes. It also inhibits leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) by inhibiting calcium channels causing induction of apoptosis as well as reduction of metastasis and angiogenesis (9).

Although alpha-tocopherol is the predominant form of vitamin E in the blood, gamma-tocopherol is more important in preventing the progression of cancer due to its biological activities (4). HT-29 is a common colon cancer cell line with various characteristics such as secretion of pro-inflammatory cytokines (TNFα, IL1, IL6), growth factors (TGF, EGF), and high cyclin D1 expression (30). Because of these properties, we used HT-29 cells to examine the effect of gamma-tocopherol in increasing 5-fluorouracil-induced apoptosis by altering the BAX/BCL2 ratio. To study the mechanism by which gamma-tocopherol induces the apoptotic effects of 5-FU, the expression of genes affecting the apoptotic pathways was investigated. Low level of apoptosis is an important step in the progression of cancer (3). The mitochondrial pathway of apoptosis is very important and is switched by regulating the expression ratio of BAX and BCL2 apoptotic proteins (12). Thus, balance in the expression of these genes is essential for cell survival and death, where the elevation of the BAX/BCL2 ratio leads to caspase-3 activity, releasing cytochrome c from the mitochondria and activation of the internal apoptotic pathway (31).

Our results were consistent with Renault et al. regarding the increase in this ratio and its effect on cell death. They showed that increasing the ratio activated caspase-3 and resulted in cell death (32). Similarly, Hongwen et al. demonstrated that a high level of this ratio reduced the proliferation of prostate cancer cells (33).

Any changes in the expression of these genes would indicate the degree of sensitivity of cells to apoptosis. In colon cancer, apoptosis plays an important role in the response to treatment, indicating a correlation between drug-induced apoptosis and treatment efficacy (10). Thus, the study of genes involved in mitochondrial apoptosis can be a predictor of resistance to chemotherapy drugs. Many studies have evaluated the function of BCL2 and BAX in colon cancer and other malignancies, indicating the role of these genes in cell survival and death (7).

Our findings showed that in vitro treatment with 5FU and gamma-tocopherol increased the BAX/BCL2 ratio. According to our results, the use of gamma-tocopherol in combination with 5-FU can be considered as a new strategy to boost the effectiveness of treatment in patients with colon cancer.

Conclusion

According to the results of the present study, a combination of 5-fluorouracil and gamma-tocopherol inhibits cell proliferation and colony formation in HT29 colon cancer cells. Correspondingly, these agents reduce cell survival synergically by increasing the BAX/BCL2 ratio. This is more effective in cells treated with a combination of 5FU and gamma-tocopherol and leads to apoptosis in chemotherapy protocols.

Conflict of Interest

The authors declared that they have no conflict of interest.

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