GENISTEIN POTENTIATES THE APOPTOTIC EFFECT OF 5-FLUOROURACYL IN COLON CANCER CELL LINES

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DAN HOTNOG1, MIRELA MIHAILA1, ANCA BOTEZATU2, GEORGIANA GABRIELA MATEI1, CAMELIA HOTNOG1, GABRIELA ANTON2, MARINELA BOSTAN1, LORELEI I. BRASOVEANU1,2

“Stefan S. Nicolau” Institute of Virology, 1Center of Immunology and 2Viral Genetics Dept., 285 Mihai Bravu Ave, S3, 030304, Bucharest, Romania
*Corresponding author: Lorelei I. Brasoveanu, “Stefan S. Nicolau” Institute of Virology, Center of Immunology, 285 Mihai Bravu Ave, S3, 030304, Bucharest, Romania, phone/fax: +40-21-3241471, e-mail: luli_brasoveanu@yahoo.com

Abstract

Transformation of normal cells, tumor progression and advanced metastasis involve a complex series of events such as genetic alterations, aberrant progression through cell cycle, induction of angiogenesis, inhibition of apoptosis and modification of cell adhesion. One of the most effective anti-cancer agents used to treat colon cancer is 5-Fluorouracyl (5-FU), but the resistance developed is an obstacle against the success of chemotherapy. Therefore, an emerging interest appeared in finding natural substances, such as genistein, for chemotherapeutic application in cancer.

The purpose of this study was to determine whether different doses of genistein might increase the effects of 5-FU treatment on apoptosis in colon cancer cells and modulate the molecules involved. Real-time cell analysis (RTCA) by xCELLigence System was used to continuously monitor the compound-mediated cytotoxicity effects in LoVo colon cancer cells. Flow cytometry assays were performed to evaluate cell cycle phases, apoptotic events and levels of nuclear antigen expression. In order to evaluate gene expression levels, isolated RNAs were reversed-transcribed, cDNAs amplified and assayed in Real Time PCR.

Data obtained showed additional effects of GST to 5-FU treatments on the increase of apoptosis, and suggested alternative approaches to obtain a stronger anti-tumor response, when use lower concentrations of anti-cancer drugs.

Keywords: colon cancer, apoptosis, cytotoxicity, genistein, 5-fluorouracyl, cell cycle, xCELLigence

Introduction

The programmed cell death, known as apoptosis, represents a cellular “suicide” mechanism which allows the control of cell number from tissues and elimination of cells having an aberrant cell cycle or present DNA mutations, cells predisposed to malignant transformation [1]. Therefore, elucidating the mechanisms of programmed cell death process seems to be of great importance for carcinogenesis, tumor evasion and with practical implications for anti-cancer therapy [2,3]. New therapeutic approaches in cancer involves restored cellular mechanisms responsible for inducing cell death (apoptosis) in tumor cells [4,5,6]. An essential step in cancer treatment is to identify the stages of development and use the basic information in prediction, prevention, early detection and development of drugs targets [2,3]. The etiology of colon cancer involves a multitude of genetic, immunological and biochemical modifications [7]. Therefore, the transformation of normal cells, tumor progression and advanced metastasis involve a complex series of events such as genetic alterations, the aberrant progression of the cell cycle, induction of angiogenesis, inhibition of apoptosis and modification of cell adhesion. Failure to eradicate the entire tumor cell
population and consequent development of resistance to chemotherapy, are the main obstacles to successful treatment of many malignant diseases, including colon cancer [8,9].

A number of oncological drugs resistant to cancer treatment, such as 5-Fluorouracil (5-FU) are increasing rapidly, possibly through the modulation of survival cell components such as proliferative or anti-apoptotic proteins [10,11,12]. Triggering apoptosis in target cells is a key mechanism by which chemotherapy promotes cell killing. Continuing efforts are made for discovering new molecular target based molecules [13,14]. In the same time, there is an emerging interest in chemotherapeutic application of natural substances, such as genistein (GST) for chemoprevention and cancer treatment. Flavonoids display a wide range of biological activities, including anti-inflammatory and cytoprotective activities and several are known to act as anti-cancer reagents [15,16].

Many anti-cancer drugs act during physiological pathways of apoptosis, leading to tumor cell destruction [17,18]. By combining flavonoids with anti-cancer drugs, an increase of the effects might be obtained, specifically in highly invasive cancer cells, while in nontumoral cells the natural compounds could reduce the cytotoxic side effects [19]. Genistein is a natural compound which occurs in Asian diet, rich in soy products [20]. It has a wide spectrum of activity, expressed both in protecting cells from malignant transformation, reducing proliferation of tumor cells and stimulating apoptosis [21-24].

The present study aimed to explore whether GST could enhance the cytotoxic effect of 5-FU against the growth of human colon cancer cell lines (LoVo) and to screen for the proper concentrations of compounds with cytotoxic or cytostatic potential, in order to overcome the chemoresistance induced by 5-fluorouracil treatment. Moreover, we investigated the possible mechanisms of interaction between 5-FU and GST regarding compound-mediated cytotoxicity, apoptosis induction, cell cycle progression of colon cancer cells, and the potential modulation of gene and antigen expression by single or combined treatments.

Materials and Methods

**Cell cultures and treatments: LoVo** human colon cancer cell line was purchased from American Type Culture Collection (ATCC) and routinely maintained in culture in RPMI-1640 medium added with 2mM L-glutamine and 10% fetal calf serum (Sigma Aldrich, St. Louis, Mo, USA), either in culture flasks or seeded in E-plates (ACEA Biosciences, USA), and incubated at 37°C/5% CO₂ humidified atmosphere. After 24h cells were treated with different concentrations of 5-fluorouracil (5-FU) and/or genistein (GST) (Sigma) for various periods of time. Then cells from flasks were detached with a non-enzymatic solution of PBS/1mM EDTA, washed twice in PBS and immediately used for: the evaluation of apoptosis events or antigen expression by flow cytometry; fixed in ice-cold ethanol/PBS (70:30) and kept until use at 4°C for cell cycle analysis; preserved as cell pellets at - 80°C for isolation of nucleic acids.

**Reagents:** The stock solutions were prepared in DMSO, and preserved at −20°C. Working drug concentrations were prepared from the stocks in culture medium before each experiment. Ribonuclease A (RNase A) from bovine pancreas, propidium iodide (PI), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), paraformaldehyde (PFA) were purchased from Sigma. Annexin V-FITC Apoptosis Detection kit and specific mouse anti-human monoclonal antibodies were purchased from Becton Dickinson (BD) Biosciences, CA, USA, while FITC-labeled rabbit anti-mouse polyclonal antibodies were purchased from Sigma.

**Real-Time Cell Analysis (RTCA):** The experiments were performed on xCELLigence System that allows cell-based in vitro assays for the assessment of cell viability
and cytotoxicity [25]. Changes in a cell status, such as cell morphology, cell adhesion, or cell viability led to a change in cell index (CI), which is a quantitative measure of cell number present in a well. Briefly, LoVo colon cancer cells were cultured in RPMI-1640 culture medium added by 2mM L-glutamine and 10% FCS and seeded in E-Plates 16 cell (ACEA Biosciences). Growth curves started to be automatically recorded on the xCELLigence System in real time. After 24 h scalar concentrations of drugs or dietary natural compounds were added, and live cells monitored.

**Cell cycle analysis:** 10⁶ of previously fixed cells were washed twice in PBS and cell pellets were resuspended in PBS. The assay was carried out using CycleTEST PLUS DNA Reagent kit and the manufacturer’s protocol from BD Biosciences. The probes were kept in the dark and at 4°C until data acquisition by flow-cytometry using a FACScan cytometer (Becton Dickinson (BD) Immunocytometry System, Mountain View, CA). Then cell cycle analyses were performed and ModFIT LT and CellFIT softwares were used to estimate the DNA index (DI) and progression through cell-cycle phases.

**Apoptosis analysis:** The apoptosis assay was carried out using the Annexin V-FITC kit and the manufacturer’s protocol from BD Biosciences. The percentages of apoptotic cells were determined by double staining with Annexin V-FITC/ PI, followed by sample analysis by flow-cytometry using WinMDI 2.9 software.

**Evaluation of nuclear antigen expression:** intracellular antigen expression was measured in cells that were first fixed for 1h/4°C in 2% PBS-PFA buffer, and permeabilized by PBS-Tween-20 (0.2%) buffer. Then washed cells were sequentially stained by specific monoclonal antibodies and FITC-labelled secondary antibodies. Cell surface fluorescence data were acquired using a FACScan flow-cytometer (BD), while data analyses were performed using WinMDI 2.9 software.

**Evaluation of gene expression:** a) **RNA isolation** - total RNAs were isolated from cell pellets using TRizol reagent (Life Technologies, USA). RNA was released maintaining its integrity, while TRizol reagent disrupted cells and dissolved cell components. Addition of chloroform, followed by centrifugation separated the solution into an aqueous phase and an organic phase. Since RNA remained exclusively in the aqueous phase, after transferring RNA was recovered by precipitation with isopropyl alcohol. The quality of isolated RNAs was assessed by measuring the concentration and purity with a Nanodrop spectrophotometer (Nanodrop Technologies, USA) and expressed as RNA integrity number (RIN) values; b) **reverse transcription** - total isolated RNA was reverse-transcribed using Promega reagents. Briefly, 2.5 µg of total isolated RNA, 0.5 µg/µl oligodT primer and 10 mM dNTPs were mixed and incubated 5 min/ 65°C. After cooling on ice, 4 µl MuMLV buffer, 100 mM DTT and 200U RNasin inhibitor and 200 U reverse transcriptase were added. Samples were incubated for 60 min/ 37°C. The polymerization reaction was stopped by heating the samples for 15 min/ 70°C. Then samples were diluted with DEPC-treated water at a final concentration of 50ng/µl and used in the amplification reaction. cDNA quality was evaluated by performing a RT-PCR assay for gliceraldehyde-3-phosphate dehydrogenase (GAPDH) house-keeping gene in all samples, using primers synthesized by Life Technologies (forward primer: 5' ACCACAGTCCATGCCATCAC 3', reverse primer: 5' TCCACCACCCTGTTTTTGCTGTA 3'), 250 ng of each cDNA was used as target while master-mix contained 1x PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 2U Taq polymerase (Promega, USA) and 400 pmol of each primer. PCR reaction was performed in a final volume of 50 µl. c) **qRT-PCR** was performed using 50 ng of each cDNA in a final volume of 50 µl containing FastStart Universal SYBR Green Master (ROX) (Roche Molecular Biochemicals, Mannheim, Germany) and 0.5 µM primers. Real-Time amplification and detection were performed using
Applied Biosystems 7300 Real Time PCR System, Foster City, CA. The amplification program consisted in an initial denaturing phase of 2 min/ 95\(^\circ\)C, followed by 40 amplification cycles (15 sec/ 95\(^\circ\)C, 30 sec specific temperature for each set of primers, 30 sec/ 72\(^\circ\)C). The threshold cycles (Ct) were recorded for the target and reference (GAPDH) genes in all the samples. Probes were tested in duplicate in each assay, and the results were quantified and double normalized using GAPDH housekeeping gene and non-treated control cells (\(\Delta\DeltaCt\)).

**Results and Discussions**

*Real time analysis of compound-mediated cytotoxicity of LoVo cells.*

The xCELLigence System of Real-Time Cell Analyzer (RTCA) allows label-free and real-time monitoring of cellular processes such as cell proliferation, cytotoxicity, adhesion, viability, invasion, and migration, using electronic cell sensor array technology. Cellular events are monitored in real time without the incorporation of labels by measuring the electrical impedance across interdigitated micro-electrodes integrated on the bottom of special tissue culture plates. In order to assess the optimal number of cells to be used in further assays, in a first line of experiments LoVo colon cancer cells were seeded at scalar densities from 10\(^3\) to 10\(^6\)/well in E-Plates for a dynamic monitoring of adherent cell proliferation. The cell growth curves were automatically recorded on the xCELLigence System in real time (data not shown). RTCA growth curves of LoVo cells and CI values prompted us to choose the 15x10\(^3\)cells/well for the compound-mediated cytotoxicity assays (Fig. 1).

<table>
<thead>
<tr>
<th>GST</th>
<th>5-FU</th>
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<tr>
<td>CTRL ; 200 uM ; 100 uM ; 50 uM ; 25 uM ; 12.5 uM ; 6.25 uM ; 3.13 uM</td>
<td>CTRL ; 200 uM ; 100 uM ; 50 uM ; 25 uM ; 12.5 uM ; 6.25 uM ; 3.13 uM</td>
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<tr>
<td>24 h ; 36 h ; 48 h ; 72 h ; 96 h</td>
<td>12 h ; 24 h ; 36 h ; 48 h ; 72 h</td>
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*Figure 1.* Proliferation vs. compound-mediated cytotoxicity curves of LoVo cells using real-time, label-free monitoring by xCELLigence Real Time Cell Analyzer (RTCA).

Therefore, we monitored compound-induced cytotoxicity to produce continuous compound- and concentration-dependent cell impedance profiles using xCELLigence System and LoVo cancer cell line as our *in vitro* models. We screened for the proper concentrations of compounds (GST and 5-FU) with cytotoxic or cytostatic potential in killing adherent tumor cells.
cells, in order to be used in further end-point assays such as evaluation of apoptotic events, cell cycle phases, antigen expression by flow-cytometry, or analysis of gene expression by Real Time PCR. Acquisition of real-time monitoring of cytotoxicity allowed for calculation of time-dependent IC50 values. Regarding timing of calculating IC50, internally, it was selected the time point where the highest concentration reached maximum response and before the controls started to crash.

In order to screen for the proper concentrations of compounds with cytotoxic or cytostatic potential, we investigated by RTCA the cytotoxicity of scalar concentrations of GST and 5-FU (from 3.13 to 200 μM), and continuously monitored the cell growth, proliferation and viability of LoVo cells. Results are expressed as normalized cell index after automatic comparison between the curves of viability for treated vs. non-treated cells (Fig. 1). After automatic data acquisition from the homogenous assays of compound-mediated cytotoxicity, we determined the compound-specific profiling, the optimal point of compound treatment and calculated the real-time IC50 values.

**Modulation of apoptotic events**

Apoptosis induction is the most potent defense against cancer, therefore chemopreventive approaches entail the use of compounds that eliminate premalignant or malignant cells by inducing them to undergo apoptosis [4].

5-Fluorouracil (5-FU) is one of the widely used chemotherapeutic drugs targeting various cancers, but its chemo-resistance remains as a major obstacle in clinical settings. In the present study, LoVo colon cancer cells were markedly sensitized to apoptosis by both 5-FU and genistein compared to the 5-FU treatment alone. When time of incubation was increased, treatments with GST and/or 5-FU had much stronger effects on the induction of apoptosis in LoVo cells. Treatment with 20 μM GST for 72 h induced higher levels of early (22.07%) and total apoptosis (37.59%) than control cells (3.59% and 9.74%, respectively).

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**Figure 2.** Effects of 24 h treatments with GST and/or 5-FU on induction of apoptosis in LoVo colon tumor cells, evaluated by Annexin-FITC/PI double staining and flow-cytometry analysis.
Moreover, combined treatments of GST with 25 μM 5-FU induced high levels of early apoptosis when 20 μM and 40 μM GST were used (37.6% and 45.84%, respectively) (Fig. 3).

**Figure 3.** Effects of 72 h treatments with GST and/or 5-FU on induction of apoptosis in LoVo colon tumor cells, evaluated by Annexin-FITC/PI double staining and flow-cytometry analysis.

Addition of flavonoids might be an alternative approach in order to obtain the same or a stronger anti-tumor response, and enhance the chemo-sensitivity of tumors to 5-FU or diminish the side-effects by using lower concentrations of anti-cancer drugs. Our results are in accordance to similar studies concerning the additive effect of GST to anti-cancer drug treatment, and in reversing the multi-drug resistance [4,5].

*Modulation of progression through cell cycle phases.*

Perturbations in cell cycle progression may account for the anti-carcinogenic effects of flavonoids. Phytochemicals that may have inhibitory and/or chemopreventive potential like GST were used in combination with cytotoxic drugs (5-FU). In order to evaluate the influence of anti-cancer drug treatment, alone or in combination with natural compounds, upon the endpoint assay of progression of colon cancer cells through cell cycle phases, LoVo cells were cultured as in the experiment described above.
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Figure 4. Flow cytometry analysis of the additional effects induced by GST on proliferation through cell cycle phases of 5-fluorouracil-treated LoVo colon tumor cells.

Our results showed that genistein significantly suppressed cell proliferation and modulated cell cycle distribution through accumulation of cells at G2/M phase. Both genistein and 5-Fluorouracil treatment induced a decrease of cells in S cell cycle phase. The results suggested that cell cycle arrest in G2M phase might represent an important cause for anti-proliferative effect of GST, as previously described (26).

Modulation of gene expression

The above results showed that genistein significantly inhibited LoVo cell proliferation and effectively induced cell cycle arrest and apoptosis in a dose- and time-dependent manner. Therefore we further examined the potential role of GST in modulation of several pro- and anti-apoptotic genes in LoVo cells. Among the several regulators of cell survival and cell death through apoptosis, p53, Bcl-2, Mdm2 and Bax are well-known primary markers.

Isolated mRNAs from treated and control cells were reverse-transcribed in cDNAs that were amplified using specific primers: a) \( p53 \) (F: 5’ GGAGCACTAAGCGAGCAGCAGT 3’/ R: 5’ CACCGATCTGAAGGGTGAAA 3’); b) \( bcl-2 \) (F: 5’ ACTTCGCCGAGATGTCCA 3’/ 5’ R: CGGTTCAGGTACTCAGTCATCC 3’); c) \( mdm2 \) (F: 5’ GGTGAGGAGCAGGCAATGT 3’/ R: 5’ GGTCTCTTCTTCCGAAGCTG 3’); d) \( bax \) (F: 5’ TGGAGCTGCAGAGGATGATTG 3’/ R: 5’ CCAGTTGAAGTGGCCGTCAGA 3’). Results were expressed as relative fold change in gene expression by \( 2^{\Delta\Delta C_t} \) values. Statistical analysis performed by Student \( t \)-test demonstrated a significant increase of relative gene expression for the proapoptotic molecules p53 when LoVo cells were treated with 20\( \mu \)M GST as compared to 25\( \mu \)M 5-FU treatment (*, \( p < 0.05 \)).
Figure 5. Real-time analysis of gene expression associated to apoptosis in LoVo cells.

In addition, a highly significant increase of $p53$ gene expression was induced by the combination of 5-FU with 40μM GST (**, $p < 0.01$). Both combinations of 5-FU with 20 or 40μM GST increased the relative gene expression of bax ($p < 0.01$).

Modulation of nuclear antigen expression

The results obtained by real-time analysis regarding gene expression prompted us to further examine the potential role of GST in modulation of nuclear antigens associated to apoptotic process in LoVo cells.

Figure 6. Flow cytometry analysis of antigen expression associated to apoptosis in LoVo cells.
Genistein, a soy-derived phytoestrogen, may have potential as a chemotherapeutic agent capable of inducing apoptosis or suppressing tumor promoting proteins. The flow-cytometry analyses showed a differential expression of constitutive nuclear antigens (Fig. 6). Treatments with 25 μM 5-FU alone or in combination with 20 or 40 μM GST seemed to increased Bax expression. In contrast, both GST and 5-FU treatments seem to decrease the expression of anti-apoptotic molecules Bcl-2 and Bclx associated to LoVo cells, behavior previously described in other colon cell lines [27,28]. Levels of P53 antigen expression seemed to be increased by single treatments with 20, 40 or 80 μM GST or by combination of 25μM 5-FU with 20μM GST (Fig. 6). Higher concentrations of GST (80 μM) used alone or combined with 5-FU decreased also Mdm-2 antigen expression.

Conclusions

Natural compounds have drawn great attention in chemoprevention and for their potential clinical application when used in combination with anti-cancer drugs. Contrast data are available on the anti-cancer effects of genistein in colon cancer. The current interest in the role of nutraceuticals in colon cancer treatment prompted us to investigate the effect of genistein on cell proliferation, apoptosis, cell cycle progression, gene and protein alterations of selected apoptosis-related proteins in LoVo cells. Using a state-of-the art technology, Real Time Cell Analysis (RTCA), we assessed the cytotoxic vs proliferative capacity of GST to modulate the chemo-sensitivity of LoVo colon cancer cells. Real-time monitoring of cellular processes by the xCELLigence Systems offered distinct and important advantages over traditional end-point assays. A comprehensive representation of entire length of the assay was possible, allowing us to make informed decisions regarding the timing of certain manipulations or treatments. The IC50 values obtained by the xCELLigence System were comparable to the IC50 value obtained by colorimetric cytotoxicity assays as MTT or XTT (data not shown).

The present study demonstrated that a combination of genistein and 5-FU can reduce cell viability in colon cancer cells. Stimuli treatment of colon cancer cells differentially induced higher levels of apoptosis as compared to untreated tumor cells, while cell cycle distribution of DNA changed. Both gene and antigen expression of molecules associated to apoptosis were modulated by single and combined treatments, and additive effects of GST to 5-FU treatment were observed. Therefore, modulatory agents of proliferation and apoptosis might be used in clinical departments in order to find new molecular targets and elaborate new therapeutic approaches for personalized treatments.

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References


