Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

Modulation of the interplay between p53, ICAM-1 and VEGF in drug-treated LoVo colon cancer cells

Received for publication, January, 22, 2017
Accepted, March, 5, 2017

CAMELIA MIA HOTNOG[1], MIRELA MIHAILA[1*], LILIANA PUIU[2], ANCA BOTEZATU[1], VIVIANA ROMAN[1], MARINELA BOSTAN[1], LORELEI IRINA BRASOVEANU[1**]**

[1] *Stefan S. Nicolau Institute of Virology, Center of Immunology, 285 Mihai Bravu Ave, Bucharest, Romania*

[2] *Prof. Dr. Alexandru Trestioreanu Institute of Oncology, 252 Fundeni Ave, Bucharest, Romania*

**Principal co-author*

***Address for correspondence to: Lorelei I. Brasoveanu, e-mail: luli_brasoveanu@yahoo.com; Marinela Bostan, e-mail: marinela.bostan@yahoo.com, "Stefan S. Nicolau" Institute of Virology, Center of Immunology, 285 Mihai Bravu Ave, S3, 030304, Bucharest, Romania, phone/ fax: +40-21-3241471*

Abstract

*Uncontrolled cellular proliferation characterizes the tumor growth and metastasis, usually being the result of multiple genetic and epigenetic insults to the cell, particularly involving proto-oncogenes and tumor suppressor genes. The angiogenesis process, inducted by a tumor, is a controlled process, influenced by angiogenic and angiostatic factors, that involves a complex interaction between tumor and endothelial cells. Tumor cell invasion and metastasis are promoted by the upregulation of angiogenic factors, such as vascular endothelial growth factor (VEGF), or of cell adhesion molecules, like intercellular adhesion molecule (ICAM-1). Angiogenesis may partially be regulated by the function of TP53, in terms of downregulation of angiogenic factor expression, whereas dysfunctional p53 stimulates angiogenesis by upregulating VEGF. Moreover, genotoxic activation of p53 leads to up-regulation of intracellular-adhesion molecule-1 (*ICAM-1*) mRNA and protein. Contrast data are available on the anti-cancer effects of nutraceuticals, like resveratrol (Rsv) and curcumin (Crm) in colon cancer, for their potential clinical application when used in combination with anti-cancer drugs. Therefore, the aim of the present study focused on the investigation of the regulatory roles of resveratrol and curcumin on cell proliferation, apoptosis, and gene expression of selected apoptosis, angiogenesis or cellular adhesion-related proteins in LoVo colon cancer cell line. The interplay between genes coding molecules associated to apoptosis, angiogenesis and adhesion were differentially modulated by single and combined treatments, and additive effects of Rsv and Crm to 5-FU treatments were observed.*

Keywords: colon cancer, p53, ICAM-1, VEGF, resveratrol, curcumin

1. Introduction

Colorectal carcinoma is one of the most common human cancers in the world and is frequently diagnosed at late stages that require chemotherapy [1]. Therefore, as a principal strategy in colon cancer treatment it has been established the multi-drug combination chemotherapy, the reason being the potential additive or synergistic produced tumor cytotoxicity [2]. Uncontrolled cellular proliferation characterizes the tumor growth and metastasis, usually being the result of multiple genetic and epigenetic insults to the cell, particularly involving proto-oncogenes and tumor suppressor genes [3]. These alterations, responsible for tumor growth and metastasis, may underlie the ability of tumors to switch to an angiogenic phenotype [4], this being a critical aspect of tumor progression. The angiogenesis process, inducted by a tumor, is a controlled process, influenced by angiogenic

and angiostatic factors that involves a complex interaction between tumor and endothelial cells [5,6].

Tumor cell invasion and metastasis are promoted by the upregulation of angiogenic factors, such as vascular endothelial growth factor (VEGF); metastasis proteins, such as matrix metalloproteinases, urokinase-type plasminogen activator, MCP-1, MIP-1, and cathepsin B; and chemokines, such as IL-8 and CXCL1 in the tumor microenvironment. In addition, the expression of adhesion molecules, such as ICAM-1 and E-selectin, is increased in tumor cells [7]. Among the many reported angiogenic factors, vascular endothelial growth factor (VEGF) is the most powerful endothelial-cell-specific mitogen that plays a key role in the complicated process of angiogenesis. It has been shown to be significantly upregulated in various human malignant tumors and to be associated with tumor angiogenesis and disease outcome [8]. In the recent years, significant progress has been made in the research on anti-angiogenic strategies for tumor therapies. Anti-angiogenic treatment may be an effective modality to potentiate immunotherapy. Tumor antigens that have been successfully targeted include epidermal growth factor receptor (EGFR), ERBB2, vascular endothelial growth factor (VEGF), cytotoxic T lymphocyte-associated antigen 4 (CTLA4), CD20, CD30 and CD52 [Scott, 2012]. Avastin, or Bevacizumab, an anti-VEGF humanized monoclonal antibody, is currently used in oncological clinics in adjuvant treatments [9].

One of the best known tumor suppressor proteins is p53, encoded by the tumor suppressor gene TP53 located on the short arm of chromosome 17 (17p13.1). The oncogenic property is due to a p53 mutation and half of all colorectal cancer cases show mutations in TP53 gene that were correlated with adenoma-to-carcinoma transitions and aggressive subsets of colorectal cancer [10]. In addition, p53 is one of the key regulators of cell cycle control and apoptosis, and tumor cells presenting p53 mutations are defective in the induction of apoptosis. Several studies indicated that angiogenesis may partially be regulated by the function of TP53. Functional p53 suppresses angiogenesis by downregulating angiogenic factor expression, whereas dysfunctional p53 stimulates angiogenesis by both upregulating VEGF and downregulating thrombospondin-1, an angiogenesis inhibitor [11].

Also the expression of several surface markers on cells involved in immune responses is regulated by p53. Among them, the cellular adhesion molecules, the intercellular adhesion molecule-1 (ICAM-1) is a crucial receptor in the cell–cell interaction, a central process to the reaction to all forms of injury. As a key endothelial receptor in the cell–cell interaction, the intercellular adhesion molecule-1 (ICAM-1 or CD54) is a well-characterized member of the immunoglobulin (Ig) gene superfamily, which binds to the β2 leukocyte integrins, leukocyte function antigen-1 (LFA-1, CD11a/CD18) and Mac-1 (CD11b/CD18) [12]. Its expression is upregulated in response to a variety of inflammatory/immune mediators, including cellular stress. Besides endothelium, ICAM-1 is also expressed in other cells, including antigen-presenting cells, where it functions as a co-stimulatory molecule for T-cell activation. In normal tissues ICAM-1 level is highest in spleen and absent in the cerebrum, peripheral nerves, pancreas, ovary, breast, uterus, cervix, prostate, lung, larynx, bone marrow, striated muscle, heart, mesothelium, esophagus, small intestine, colon and liver. In addition, ICAM-1 is associated with a variety of cancer types and appears to play a role in cancer metastasis, as a biomarker or target for therapeutic interventions [13]. Additional ICAM-1 ligands are mucins (e.g. MUC-1) or E-cadherin. ICAM-1 is induced by cytokines and various stress stimuli such as hypoxia, ultraviolet and ionizing radiation [12]. Genotoxic activation of p53 leads to up-regulation of intracellular-adhesion molecule-1 (*ICAM-1*) mRNA and protein. Previous studies demonstrated a NF-κB-independent role for p53 in ICAM-1 regulation that may link p53 to ICAM-1 function in various physiological and pathological settings [12].

Apoptosis, also known as “programmed cell death”, is the most potent defense against cancer, and represents a cellular mechanism that allows the control of cell number from tissues and elimination of cells that present DNA mutations or have an aberrant cell cycle, predisposed to malignant transformation [14]. Apoptosis, the cellular intrinsic death program, plays a crucial role in the regulation of tissue homeostasis; an imbalance between cell death and proliferation may result in tumor formation [15].

Targeted therapies and cancer immunotherapies are two novel treatment modalities that have recently begun to enter into oncological clinics, that offer a number of possible synergies in treatment when used together, but there are more aspects to be further studied. Many tumor targeted therapies affect pathways that are also crucial for immune development and function, which suggests the possibility that targeted therapies may help to optimize anti-tumor immune responses from immunotherapies. Similarly, immunotherapies may serve to consolidate impressive clinical responses from targeted therapies into long-lasting clinical remissions [8]. Important considerations regarding optimization of dose, sequence and timing of targeted therapies will be required when rationally designing future clinical trials, in order to maximize anti-tumor efficacy while minimizing any immunosuppressive side effects [9].

Recent studies focused on the discovery of new chemotherapeutic agents among natural products, since many plants and their bioactive compounds displayed anti-carcinogenic and anti-proliferative effects towards colon cancer cells [16-18]. Positive correlations between antioxidant activities of plants and their anti-proliferative effects, suggesting the potential action of antioxidants in inhibiting cancer cell growth were also reported [19,20]. Contrast data emphasizes the growing role of diet and use of natural active compounds as modulators of immunological processes in cancer therapy. Among them, resveratrol and curcumin are intensely used as additives in colon cancer prevention and treatments.

Resveratrol (Rsv, trans-3,4',5-trihydroxystilbene), a naturally occurring polyphenol phytoalexin, is abundant in a wide variety of plants and their products, including grapes and red wine, mulberries, peanuts, seeds, and has anti-inflammatory, antioxidant, anti-neoplastic, anti-carcinogenic, anti-tumorigenic, cardioprotective, neuroprotective, anti-aging and antiviral effects [21-23]. Resveratrol exhibited anti-colon cancer properties by inhibiting cell proliferation, inducing apoptosis, decreasing angiogenesis, and causing cell cycle arrest [24-26]. Curcumin (CRM, 1,7-bis-(4- hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a diarylheptanoid and the principal curcuminoid of turmeric, extracted from *Curcuma longa;* it possesses anti-inflammatory and antioxidant properties, and has a strong inhibitory effect on cell proliferation in the HT-29 and HCT-15 human colon cancer cell lines [27-28].

Our previous studies on the potential roles of natural compounds in cancer therapy showed a modulatory role of resveratrol in proliferation and apoptosis of colon [18] and breast [29] cancer cells. Therefore, in the present study, we have further investigated the effect of added bioactive compounds like the nutraceuticals Rsv or Crm to anti-cancer drugs and/or anti-angiogenic therapy on proliferation, apoptosis and gene expression related to angiogenesis in LoVo colon cancer cell line.

2. Materials and methods

2.1. Reagents: Resveratrol ((3,5,4'-trihydroxystilbene, Rsv), 5-fluorouracil (5-FU), Oxaliplatin (OxlPt), Cisplatin (CisPt), dimethyl sulfoxide (DMSO), paraformaldehyde (PFA) were purchased from Sigma. High concentrated stock solutions were prepared as recommended, in ultrapure sterile water (CisPt, OxlPt) or DMSO. Avastin was purchased from Roche Pharmaceuticals (Suisse). Working drug concentrations were prepared from the

stocks in complete culture medium before each experiment. Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit for flow-cytometry was purchased from Becton Dickinson (BD) Biosciences, Mountain View, CA, USA, while Total ROS Detection Kit for flow cytometry was obtained from ENZO Life Sciences, Farmingdale, NY, USA.

2.2. Cell cultures and treatments: LoVo cell line, derived from Dukes' type C, grade IV, colorectal adenocarcinoma was purchased from American Type Culture Collection (ATCC). Adherent cells were routinely maintained in culture in DMEM:F12 medium added by 2mM L-glutamine and 10% fetal bovine serum (Sigma Aldrich, St. Louis, Mo, USA), and incubated at 37^oC/ 5% CO_2 humified atmosphere. After 24h of culture, when cells achieved 50-70% confluency, cultures were treated with different concentrations of either anti-cancer drugs (5-FU, OxlPt), anti-angiogenic Avastin or RSV, or with various combinations for different periods of time. Then cells were detached with a nonenzymatic solution of PBS/1mM EDTA, washed twice in PBS and immediately used for the evaluation of apoptosis events or ROS levels by flow-cytometry.

2.3. Real-Time Cell Analysis (RTCA): The experiments were performed on xCELLigence DP-System, that allows cell-based *in vitro* assays for the assessment of cell viability and cytotoxicity [30]. Changes in cell status, such as cell adhesion, cell morphology, or cell viability lead to a change in cell index (CI), which is a quantitative measure of cell number present in a well. Briefly, $15x10^3$ LoVo colon cancer cells were seeded in 100 ul culture medium in each well of E-Plates 16 cell (ACEA Biosciences), and after 10 min for equilibration of cell culture, plates were put in the xCELLigence DP-System, and placed in a 5% CO_2 humidified incubator. Growth curves started to be automatically recorded on the xCELLigence System in real time. When cell proliferation reached a CI (cell index) value over 1.0, scalar concentrations of drugs and/or dietary natural compounds were added, and live cells monitored.

2.4. 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 evaluated by Annexin V-FITC/PI double staining, followed by sample analysis by flow-cytometry using WinMDI 2.9 software.

2.5. Isolation of nucleic acids: a) RNA isolation - total RNAs were isolated from cell pellets using TRIzol reagent (Life Technologies). 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 evaluation of concentration and purity using Nanodrop spectrophotometer (Nanodrop Technologies, USA) and RIN (RNA Integrity Number) values; **b) Reverse transcription** - total isolated RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Briefly, total isolated RNA (2 μg for each sample) was added to a reaction mix which contains: RT-buffer, RT-Random Primers, dNTP Mix, MultiScribe™ Reverse Transcriptase and DEPC-treated water in a 20 μl total volume. The samples were loaded in a thermocycler and the reaction took place in the following conditions: 10 min/ 25^0C, 120 min/ 37^0C and 5 min/ 85^0C. Then the samples were diluted with DEPC-treated water at a final concentration of 10ng/μl and used in the amplification reaction. cDNA quality was evaluated by performing a RT-PCR assay for gliceraldehyde-3-phosphate dehydrogenase (GADPH) house-keeping gene in all samples.

2.6. Real-Time PCR TaqMan Assays: PCR reaction was performed in a final volume of 50 μl and the cycling conditions were as follows: 50^0C/2min, 95^0C/10min and 95^0C/30sec,

60^0C/30sec for 40 cycles. 50 ng of total cDNA was amplified in a 20 µl total volume reaction, using ChromoFour Real Time PCR System (Biorad). TaqMan probes for target genes (TP53 Hs01034249_m1; BAX Hs00180269_m1; ICAM1 Hs00164932_m1; VEGFA Hs00900055_m1) and endogenous control gene (GAPDH, Hs99999905_m1) were provided by Applied Biosystems as custom assays. To check for amplicon contamination, every run contained a negative control in which nuclease-free water was a substitute for template. The threshold cycles (Ct) were recorded for the target genes and reference (GAPDH) in all the samples. Samples were tested in duplicate in each assay, and the results were quantified and double normalized using GAPDH house keeping gene and non-treated control cells (□□Ct).

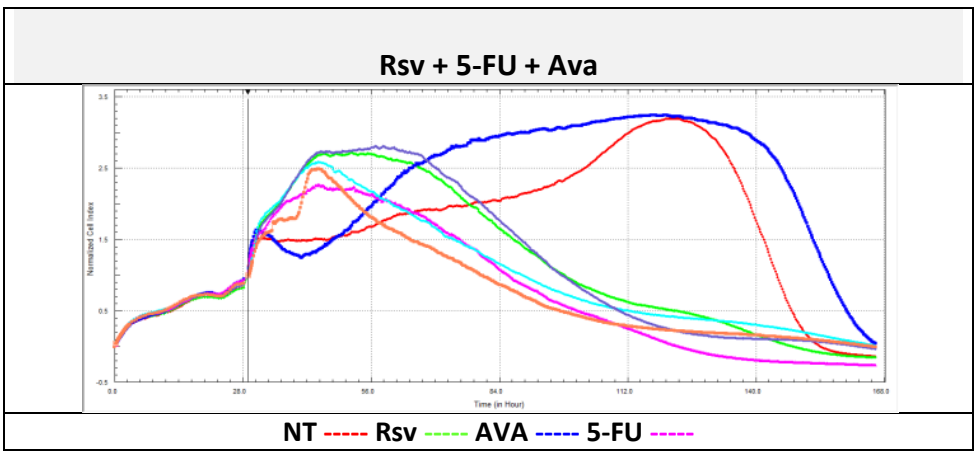
2.7. Statistical Analysis was performed using Student *t*' and ANOVA tests; *p* values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Modulation of cell proliferation by natural compounds in drug-treated colon cancer cells

We have previously demonstrated the modulatory role of Rsv on proliferation of anti-cancer drug treated cancer cells [18, 29]. In the present study we have further investigated the effect of adding bioactive compounds like Rsv or Crm on proliferation of colon cancer cells when anti-angiogenic therapy with Avastin was added to anti-cancer drug therapy with 5-FU. To analyze the proliferation profiles of treated LoVo cells, we continuously monitored cell growth by using real time cells analysis (RTCA) assay and xCELLigence system. Real-time impedance data obtained were used to generate compound-specific profiles that are dependent on the biological mechanisms of action of each used compound.

LoVo cancer cells were seeded in E-plates 16 in complete culture medium ($15x10^3$ cells/well/100ul), plates put in xCELLigence DP device and cultured at 37^0C/5% CO2 humified atmosphere. After 24h, cells were added by single or combined treatments of 5-FU, Ava or natural compounds (Rsv or Crm), and growth curves were registered in real time on computer by using RTCA 2.1.2. Software (Fig. 1). The inhibition of the proliferative capacity of single or combined treatments with 25 uM anti-cancer drugs (5-FU), 100 ug/ml of anti-angiogenic Ava, 50 uM of Crm or Rsv were investigated in LoVo colon cancer cell line in order to modulate the chemo-sensitivity of colon cancer cells to drug treatments, and overcome the chemo-resistance (Fig. 1). Acquisition of real-time monitoring of cytotoxicity allowed for calculation of time-dependent IC50 values. Using xCELLigence System and LoVo cancer cell line we obtained continuous compound-dependent cell impedance profiles as our *in vitro* models (Fig. 1).



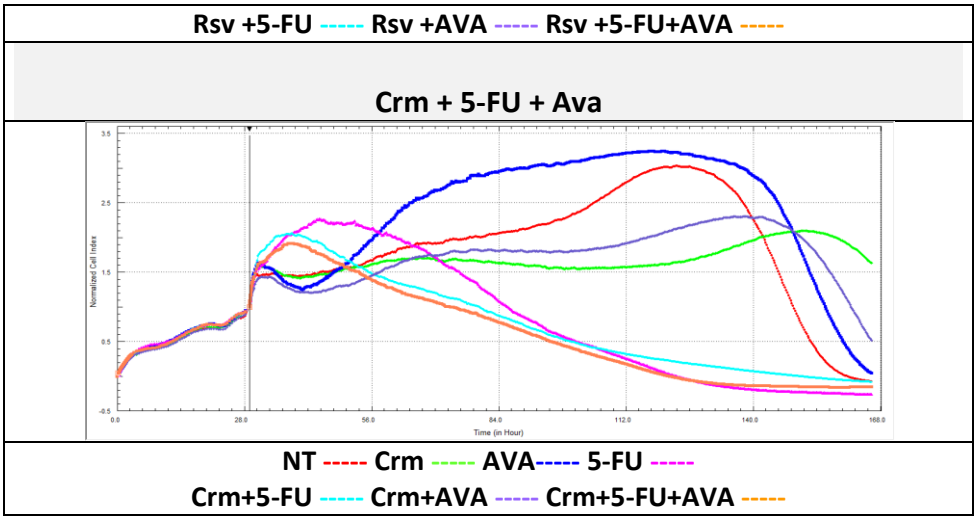


Figure 1. Modulation by Rsv or Crm of cell proliferation of drug-treated LoVo cells.

Screening for the proper combinations of compounds with cytotoxic or cytostatic potential in inhibiting the growth of adherent tumor cells, made possible the choice of the proper combinations to be further used in end-point assays such as evaluation of apoptosis or evaluation of gene expression.

3.2. Evaluation of apoptotic events induced by 5-FU and/or anti-angiogenic treatments in LoVo cells

The control of cell number from tissues and elimination of those predisposed to malignant transformation, having an aberrant cell cycle or presenting DNA mutations, might be performed by apoptosis, a cellular "suicide" mechanism or programmed cell death [15]. The mechanisms of apoptosis might be of great importance for carcinogenesis, tumor evasion, and have practical implications for anti-cancer therapy, since many anti-cancer drugs act during the physiological pathways of apoptosis, in the end leading to tumor cell destruction [14, 31].

In order to evaluate the potential role of Rsv or Crm in the modulation of the apoptosis induced by anti-cancer therapy, added or not by anti-VEGF therapy, the apoptotic events were evaluated by flow-cytometry.

LoVo colon cancer cells were cultured in complete medium for 24h, culture medium changed, and cells sensitized for additional 24h with 50 uM Rsv in the presence or absence of 25 uM 5-FU, and/or 100 ug/ml Ava. Alternatively, 100 ug/ml Ava were added to 25 uM 5-FU. Then LoVo cells were detached with PBS/1mM EDTA, sequentially washed with PBS, and Wash Buffer, and centrifuged 5 min/300xg. Pellets were suspended in 400 ul Binding Buffer, and 100 ul distributed in flow tubes and stained with 5 ul of Annexin-V/FITC and/or PI for 15 min/RT at 37^{o}C in the dark. The green and red fluorescences were measured by using FACSCantoII flow-cytometer and DIVA and WinMDI2.9 softwares.

LoVo live cells were not labelled with Anexin-V/FITC or PI; early apoptotic cells displayed green fluorescence; late apoptotic cells were double stained, while necrotic cells labelled only PI. Total apoptosis was calculated by summing early and late apoptotic events. Figure 2 shows significant experiments of modulated apoptosis by 24h single or combined treatments.

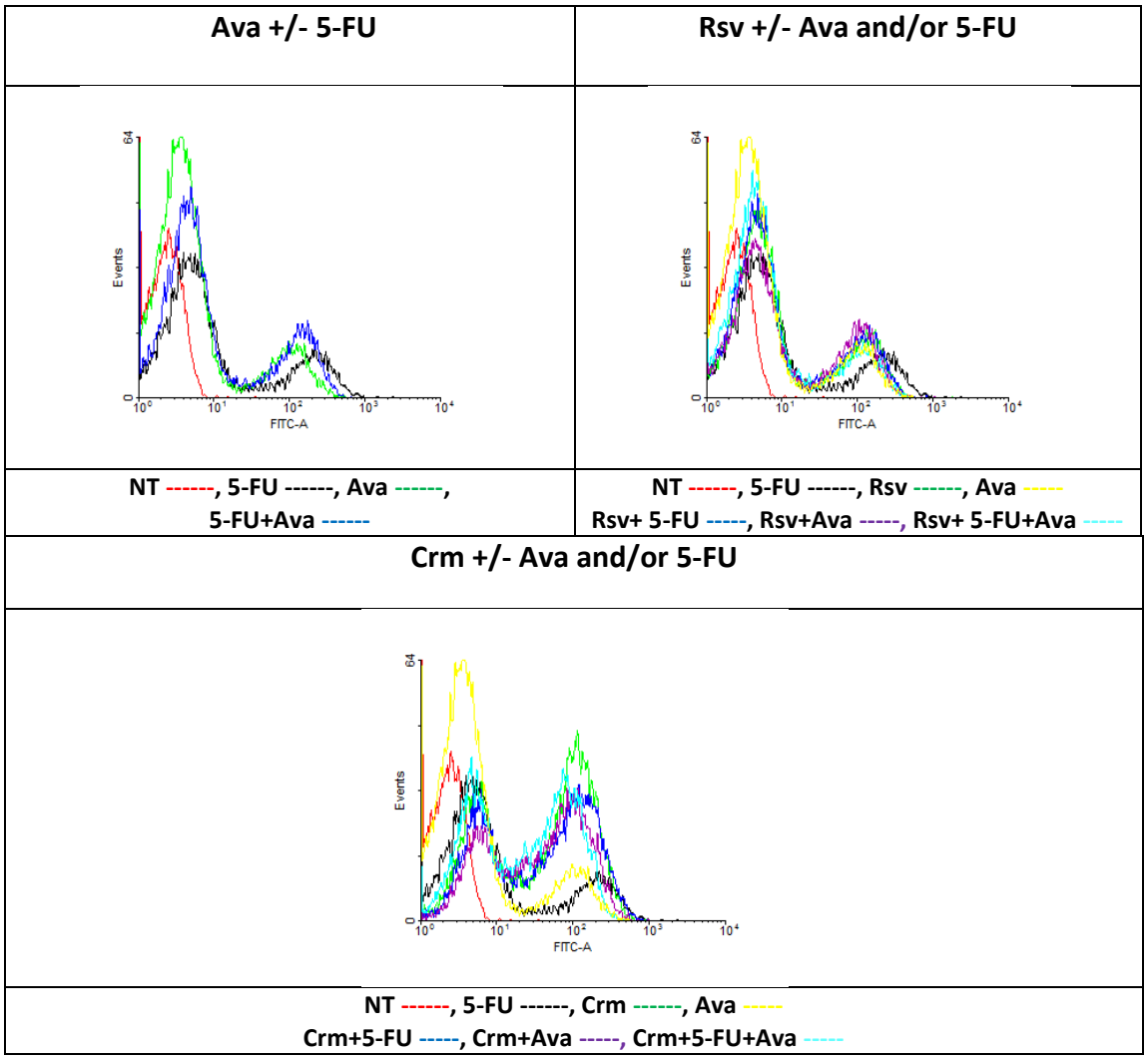


Figure 2. Representative histograms for apoptotic events induced by 5-FU, Ava and/or Rsv or Crm

All treatments induced an increase of both early and late apoptotic levels (Table 1). Natural compouds displayed a similar behaviour. RSV had an additive role in modulating apoptosis induced by 5-FU: early apoptotic levels increased from 14.6% to 21.6%, while total apoptotic levels increased from 23.7% to 31.2%. (Table 1).

Table 1: Percentages of apoptotic events induced by single or combined Avastin anti-angiogenic treatments LoVo colon cell line

Compound	Early Apoptosis	Late Apoptosis	Total Apoptosis
NT	**1.7**	**1.4**	**3.1**
5-FU	**14.6**	**9.1**	**23.7**
Ava	**11.5**	**3.7**	**15.2**

5-FU+Ava	**15.7**	**9.2**	**24.9**
Rsv	**14.9**	**8.8**	**23.7**
5-FU+ Rsv	**21.6**	**9.6**	**31.2**
Rsv+Ava	**10.6**	**8**	**18.6**
5-FU+Ava+Rsv	**20.8**	**8.5**	**29.3**
Crm	**30.1**	**6.2**	**36.3**
5-FU+ Crm	**37.8**	**9.3**	**47.1**
Crm +Ava	**39.7**	**5.5**	**46.2**
5-FU+Ava+ Crm	**41.9**	**12.4**	**54.3**

Crm increased the apoptosis induced by 5-FU: early apoptotic levels increased from 14.6% to 30.1%, while total apoptotic levels increased from 23.7% to 36.3%. Crm had an additive role in increasing also the early apoptosis induced by Ava treatment, from 11.5% to 39.7%. The highest levels of apoptosis were induced by combination of Crm both with 5-FU and Ava (Table 1).

3.5. Modulation of gene expression

Despite many decades of research, the mechanisms underlying chemoresistance are still poorly understood. There is growing evidence that the inflammatory tumor microenvironment modulates not only cancer development but also cancer responsiveness and resistance to conventional anticancer therapies The above results showed that resveratrol significantly inhibited LoVo cell proliferation and effectively modulate apoptosis in a dose- and time-dependent manner. Therefore we further examined the potential role of Rsv and Crm in modulation of several genes related to apoptosis, angiogenesis and adhesion processes. Among the several regulators of cell survival and cell death through apoptosis, p53 and Bax are well-known primary markers [32].

Therefore, isolated mRNAs from treated and control cells were reverse-transcribed in cDNAs, that were amplified using specific optimized primers for Taqman RT-PCR assay.

Statistical analysis performed by Student *t'* test demonstrated a significant increase of relative gene expression for the proapoptotic p53 when LoVo cells were treated with Rsv or the combinations of Rsv or Crm with 5-FU, as compared to 25μM 5-FU treatment (*, $p < 0.05$). In addition, a highly significant increase of *Bax* gene expression was induced by the combination of 5-FU with 50 uM Crm (**, $p < 0.01$) (Fig. 3).

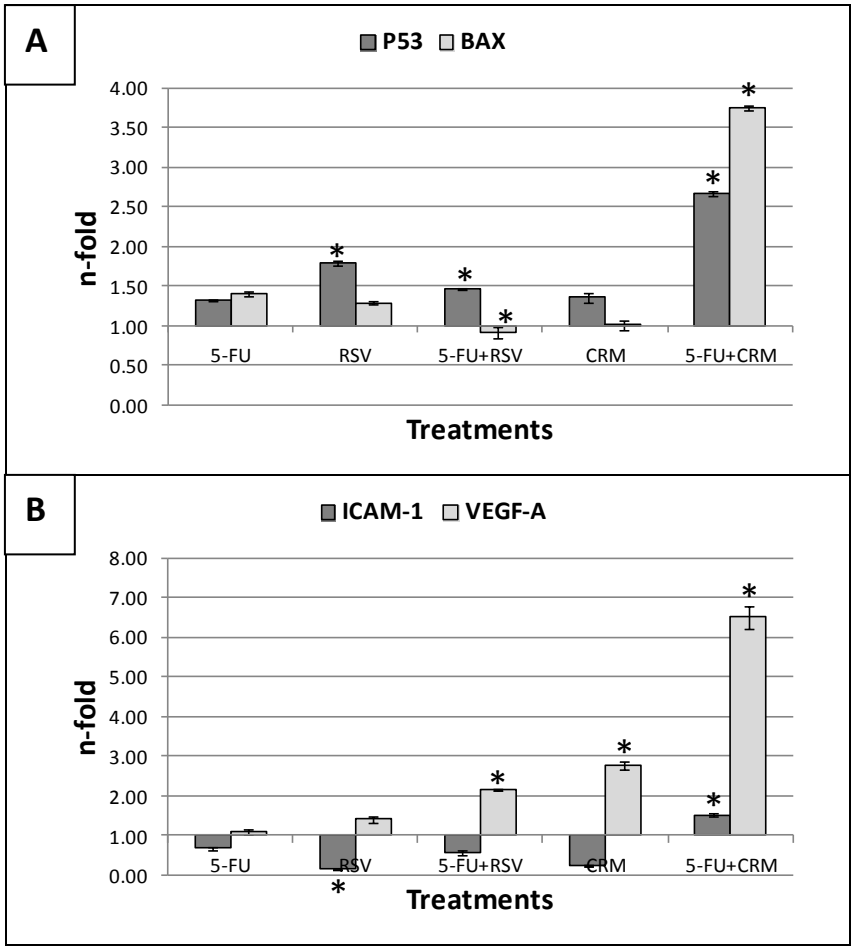


Figure 3. Levels of modulated gene expression.
Results were expressed as relative fold change in gene expression by $2^{-\Delta\Delta}$Ct values (n-fold).
Statistical analysis was made by comparing all treatments to 5-FU modulatory effect
(*, $p < 0.05$) in both panels, A) for TP53 and Bax genes, and B) for ICAM-1 and VEGF-A genes.

ICAM-1 gene expression was decreased by all treatments, excepting the combination of 5-FU with Crm. Both combinations of 5-FU with Rsv or Crm increased the relative gene expression of VEGF ($p < 0.01$) (Fig. 3).

Previous studies showed that resistance of colon cancer cells to 5-FU chemotherapy exposure is correlated with Bax and p53 status [33]. All the obtained data are sustained by previous observations concerning the interplay between p53 and VEGF or ICAM-1. Moreover, two central pathways for the hypoxic response in cancers are regulated by (1) the major tumor suppressor p53; and (2) the crucial modulator of new vasculature, 'neo-angiogenesis', vascular endothelial growth factor (VEGF). The consequences for tumor vascularization of disrupting these pathways has important implications for p53-targeted anti-cancer therapy [11]. In addition, also the p53/ICAM-1 relationship may be important for immune surveillance since activation of ICAM-1 by p53 has been implicated in leukocyte infiltration during tumor-targeted inflammation, suggesting intercellular as well as intracellular guardian roles for p53 [10]. Regarding the second point, the apparent p53–ICAM-1 link suggests either that ICAM-1 may participate in p53-dependent cellular processes such as growth arrest and apoptosis, and/or that p53 may have a role in certain inflammatory conditions. It is well

documented that cellular interactions influence a variety of signalling events including those engaged in survival [12].

Conclusions

Many anti-cancer drugs act during physiological pathways of apoptosis, leading to tumor cell destruction. By combining natural compounds with anti-cancer drugs, an increase of their effects might be obtained, specifically in highly invasive cancer cells, while in nontumoral cells the natural compounds could reduce the cytotoxic side effects [34,35].

Nutraceuticals have drawn great attention in chemoprevention and for their potential clinical application when used in combination with anti-cancer drugs [36]. Contrast data are available on the anti-cancer effects of resveratrol and curcumin in colon cancer [37]. The current interest in the role of nutraceuticals in colon cancer treatment prompted us to investigate the effect of resveratrol and curcumin on cell proliferation, apoptosis, and gene expression of selected apoptosis, angiogenesis or cellular adhesion-related proteins in LoVo cells. Using a state-of-the art technology, Real Time Cell Analysis, we assessed the capacity of Rsv or Crm to modulate the chemo-sensitivity of 5-fluorouracyl and/or Ava treated LoVo colon cancer cells. Stimuli treatment of colon cancer cells differentially induced higher levels of apoptosis as compared to untreated tumour cells. The interplay between genes coding molecules associated to apoptosis, angiogenesis and adhesion were modulated by single and combined treatments, and additive effects of Rsv and Crm to 5-FU treatments 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.

Acknowledgments

We are grateful for the support offered by the POS-CCE O2.2.1. 433/ 2012 project.

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