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EXPERIMENTAL STUDY

# Synergic and comparative effect of 5-fluorouracil and leucoverin on breast and colon cancer cells through TRPM2 channels

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**ABSTRACT**
OBJECTIVES: We aimed to reveal the role of 5-fluorouracil (5-FU) and Leucovorin (LV) along with transient receptor potential protein melastatin 2 (TRPM2) channels in breast and colon cancer cells during the treatment process.
BACKGROUND: 5-FU and LV are widely used in breast and colon cancers for chemotherapy. It has been reported that the expression of TRPM2 channels increased intensively in cancer cells.
METHODS: Breast (MCF7) and colon (Caco-2) cells were cultured and divided into seven main groups. The cells in the group were incubated with 5-FU and LV for 24 hrs and then incubated with Antranilic acid. The effects of medicines were investigated on all molecular pathways of apoptosis.
RESULTS: It was found that 5FU and LCV, administered separately and together on breast cancer cell culture and colon cancer cell culture increased the intracellular calcium levels by stimulation of TRPM2 channels in both cancer cells.
CONCLUSION: As the result of our study, it has been shown that apoptotic effects of 5FU and LV on both colon and breast cancer cells were directly related to TRPM2 channels and that TRPM2 channels played an important role in the whole molecular pathway of apoptosis leading to increased intracellular $Ca^{2+}$ ($Ca^{2+}$) levels and increased mitochondrial depolarisation *(Fig. 6, Ref. 43*). Text in PDF *www.elis.sk.*
KEY WORDS: breast and colorectal cancers, TRPM2 channel, 5-flourouracil, leucoverin, oxidative stress.

## Introduction

Breast and colon cancers are common and are among the major causes of death worldwide. Breast cancer, along with lung cancer, is the one of the leading causes of death among women (1). Colon cancer is the third most common cancer in men after lung and prostate cancers (2). Chemotherapy in breast and colon cancer is widely used during preoperative and postoperative period. As the result of recent studies, significant progress has been made in the treatment with the development of chemotherapy strategies that are applied according to various cancer subtypes and stages, especially in breast cancer (3). 5-fluorouracil (5-FU) is widely used in breast cancers and digestive tract cancers, especially in colon cancers because of its relatively high efficiency and relatively low cost (4). 5-FU is generally used in combination with leucovorin (LV) in clinical chemotherapy applications (5-FU/LV).

Transient receptor potential (TRP) protein was first identified in Drosophila trp genes in 1989 (5). The TRP protein family contains different groups of calcium ion ($Ca^{2+}$) permeable and non-selective cation channels and is found in many living organisms (6, 7, 8). There are 6 subtypes of TRP channels; TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystic kidney disease), TRPML (mucolipin) and TRPA (ankyrin) TRP channels contain tetrameric subunit stoichiometry and each subunit contains cytoplasmic N and C-terminal regions, 6 transmembrane (TM) domains, and TM5-TM6 interspaces (9). TRP channels are responsive to stimuli such as receptor stimulation, heat, plant-derived compounds, environmental irritants, mechanical stress, pH, intra- and extracellular voltage, and respond physiologically and pathologically (5, 6, 7–10). In addition, TRP channels respond fairly well to oxidative stress mediators such as: reactive oxygen species (ROS), reactive nitrogen species (RNS), and other electrophiles (11–14).

Transient receptor potential melastatin-2 (TRPM2) channels have been reported to have significant effects on migration and cell death in tumour and immune system cells (15). TRPM2 protein has been shown to induce cell death by cytokine and bacterial peptide activation, directly through cell migration and oxidative stress (16). TRPM2 channels have been reported to increase expression intensively in head and neck, bladder, liver, and lung adenocarcinomas, particularly breast cancer (17).

Intracellular $Ca^{2+}$ is involved in the activation of TRP channels through receptor stimulation and enzymes such as the $Ca^{2+}$ dependent protein phosphatase, therefore it plays an important role in oxidative stress and apoptosis (5,18). It is known that chemotherapeutic agents increase intracellular $Ca^{2+}$ levels by free oxygen radi-

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cals in the cell and lead to oxidative stress. It has been reported that 5-FU induces oxidative stress by increasing free oxygen radicals in the cell and causes apoptotic effects in the antioxidant defence system resulting in damage and repression (19, 20).

Based on this information, we aimed to investigate the role of TRPM2 channels, which are known to be susceptible to oxidative stress in breast and colon cancer cells, by administering 5-FU and LV on colon and breast cancer cell colonies, both separately and in combination, and to elucidate the effects of these drugs on TRPM2 channels and intracellular $Ca^{2+}$ levels.

## Materials and methods

### *Cell culture*

MCF7 and Caco-2 cell line was purchased from Culture Collection of Animal Cells, Foot and Mouth Disease (ŞAP) Institute, Ankara, Turkey. MCF7 cells were cultured in RPMI 1640 medium and Caco-2 cells were cultured in DMEM (Dulbecco's modified Eagle's medium. All mediums containing 10 % foetal bovine serum (FBS) (Fisher Scientific, and 1 % penicillin/streptomycin (Biochrom, Berlin, Germany). The cells were seeded in 8-10 flasks at a density of $1\times10^6$ cells per flask (filter cap, sterile, 5 ml, 25 $cm^2$). Cells were incubated in T25 flasks at 37 °C at 5 % $CO_2$ in a humidified incubator. After the cells have reached 75–85 % confluence, the cells were incubated with the chemical compounds described in the groups section. The cells were examined daily for evidence of contamination. After treatments, the cells were detached with 0.25 % Trypsin–EDTA and split into the sterile falcon tubes for analysis.

### *Reagents*

Roswell Park Memorial Institute (RPMI) 1640 Medium, Dulbecco's modified Eagle's medium (DMEM), Trypsin–EDTA, Antranilic acid, Fetal Bovine Serum and penicillin-streptomycine, cumenhydroperoxyde and Dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical (St. Louis, MO), Fura-2/AM was obtained from Calbiochem (Darmstadt, Germany). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was purchased from Thermo Fischer (Waltham, MA USA). Dihydrorhodamine-123 (DHR 123) obtained from Molecular Probes (Eugene, OR, USA). Caspase 3 and Caspase 9 substrates [N-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (ACLEHD-AMC) and N-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC)] were purchased from Biovision (San Francisco, USA). APOPercentage dye with releasing buffer were purchased from Biocolor (Belfast, Northern Ireland) A mitochondrial stain 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) was purchased from Santa Cruz (Dallas, Texas, USA).

### *Study groups*

MCF7 and Caco-2 cells were cultured at 37 °C. The cells were divided into seven main groups.

Group 1 (Control): The cells were not incubated with 5FU, Leucoverin and antranilic acid (ACA), but were kept in a flask containing the same cell culture medium and conditions for 72 h.

Group 2 (5FU): Cells in the group were incubated with 5 μM 5-Fluorouracil for 24 hrs (21).

Group 3 (5FU+ACA): Cells in the group were incubated with 5 μM 5-Fluorouracil for 24 hrs and then incubated with Antranilic acid (ACA, 0.04 mM, 30 min).

Group 4 (5FU+LCV): Cells in the group were incubated with 5 μM 5-Fluorouracil for 24 hrs and then incubated with 2 μM Folinic acid (Leucoverin) for 24 hrs.

Group 5 (5FU+LCV+ACA): Cells in the group were incubated with 5 μM 5-Fluorouracil for 24 hrs and then incubated with 2 μM Folinic acid (Leucoverin) for 24 hrs and then incubated with Antranilic acid (ACA, 0.04 mM, 30 min).

Group 6 (LCV): Cells in the group were incubated with 2 μM Folinic acid (Leucoverin) for 24 hrs (22).

Group 7 (LCV+ACA): Cells in the group were incubated with 2 μM Folinic acid (Leucoverin) for 24 hrs and then incubated with Antranilic acid (ACA, 0.04 mM, 30 min).

In related experiments (except for calcium signalling), the cells were further treated with Cumen hydroperoxyde (CMPx) (0.1 mM, 10 min) for activation of TRPM2 channel before related analysis and they were also inhibited by the TRPM2 channel blocker ACA (0.04 mM, 30 min) before related analysis in the presence of normal extracellular calcium (1.2 mM). During calcium signalling analysis (Fura-2/AM), cells were stimulated on 20th cycles with 0.1 mM CMPx in the presence of normal extracellular calcium (1.2 mM).

### *Measurement of intracellular free calcium concentration ($[Ca^{2+}]i$)*

The $Ca^{2+}$ i concentration was measured by UV light-excitable Fura-2 acetoxymethyl ester (Fura-2-AM) as an intracellular calcium ion indicator. After cell were incubated with the chemical compounds described in groups section, cells were detached with 0.25 % Trypsin–EDTA from T25 flasks. After centrifuged (100 G, 5 min), the medium was removed and replaced with HEPES-buffered saline [HBS; 5 mM KCl, 145 mM NaCl, 10 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, (1.2 mM). 10 mM HEPES and 0.1 % (w/v) bovine serum albumin (BSA); pH 7.4] containing 5 μM fura-2 AM and 0.05 % (w/v) Pluronic F-127, and cells were incubated for 1 h at 37 °C in the dark. The loaded cells were washed twice with HBS and covered with 1000 μL of HBS supplemented with 2.5 mM probenecid for at least 20 min at 37 °C in the dark to allow for Fura-2 AM de-esterification. Cells were seeded in clear flat-bottom black 96-well culture trays (Grainer Cell Star, Life Sciences USA) at a density of $3\times10^4$ cells/per well. Fluorescence emission intensity at 510 nm was determined in individual wells using a plate reader equipped with an automated injection system (SynergyTM H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles (cycle: 3 s; exposure: 25 flashes; gain: 120) in response to agonists (CMPx, 0.1 mM) added with the automated injector. $[Ca^{2+}]i$ in cells was expressed as the average emission at 510 nm in individual wells in response to excitation at 340/380 nm normalized to initial fluorescence emission obtained during the first 10–20 cycles. Measurement of $[Ca^{2+}]i$ was performed as modified by Uguz et al, 2009 and Martinez et al (23, 24).

*Intracellular ROS production measurement*

Rhodamine 123(Rh 123) is a non-fluorescent, non-charged dye, which is cell membrane permanent green florescent dye, which can easily pass the cell membranes, where it is oxidized to cationic rhodamine 123, which is localised in the mitochondria and exhibits green fluorescence. It was sequestered by mitochondria. The cells ($10^6$ cells/ml for per group) were incubated with 20 µm DHR 123 as florescent oxidant dye at 37 °C for 25 min (25). The Rh123 fluorescence intensities were determined by using an automatic microplate reader (Synergy™ H1, Biotek, USA). Excitation and emission wavelengths of the analyses were 488 nm and 543 nm, respectively. Data are presented as fold-increase over the pre-treatment level.

*Apoptosis assay*

The APOPercentageTM, which is used as an assay for the detection and quantification of apoptosis (Biocolor Ltd., Belfast, Northern Ireland) was performed according to the manufacturer instructions. The APOPercentageTM assay is a dye-uptake assay, which stains only the apoptotic cells with a red dye. When the membrane of apoptotic cell lost its asymmetry, the APOPercentage dye is bonded with phosphatidyl serine lipids actively and transported into cells, staining apoptotic cells red, thus allowing the detection of apoptosis by a multiplate reader as previously described, elsewhere (26). After the cells were incubated with the chemical compounds described in the groups section, the cells were washed with 1xPBS and resuspended in 50 µl 1xPBS for per group. After added 950 µl 1xPBS and 10 µl APOPecerntage dye (Biocolor, Belfast, Northern Ireland), than cells were incubated on shaker for 30 min at 37 °C in a humidified $CO_2$ incubator in the dark. After the incubation, cells were washed twice by re-suspension in 500 µl of 1xPBS and centrifugation for 5 min at 1x100 g. After removing the supernatant, 200 µl apopercentage release

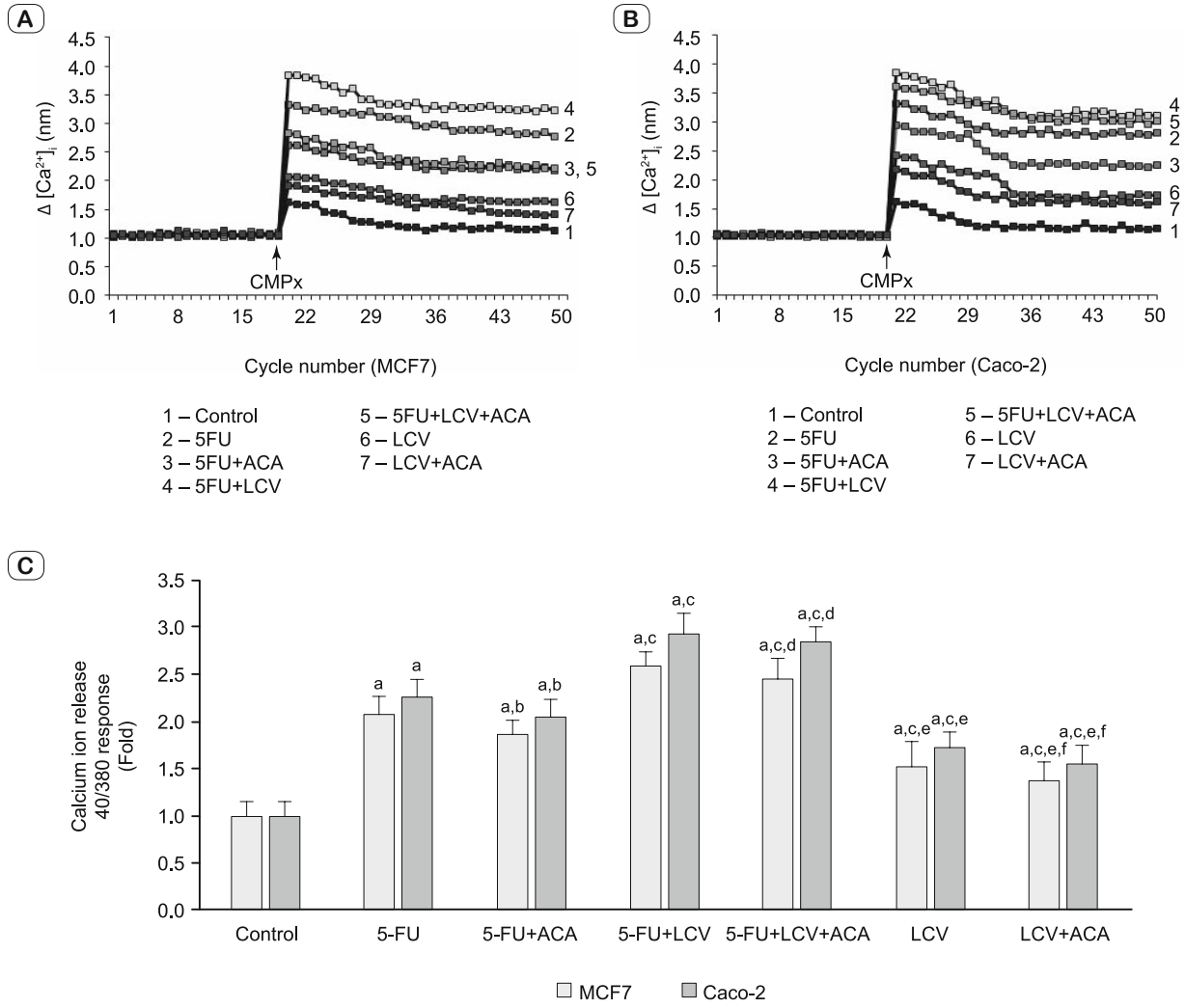


**Fig. 1. The effect of 5-FU (5 µM, 24 hrs) and Leucoverin (2 µM, 24 hrs) on $[Ca^{2+}]i$ through TRPM2 channels in MCF7 (A) and Caco-2 (B) cells and cellular calcium ion release (fold) (C). Cells are stimulated by Cumene hydroperoxide (CMPx 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (ACA 0.04 mM for 30 min) (mean ± SD and n = 10). $^{a}p < 0.001$ versus control group, $^{b}p < 0.05$ and $^{c}p < 0.001$ versus 5FU group, $^{d}p < 0.05$ and $^{e}p < 0.001$ versus 5-FU+LCV group, $^{f}p < 0.05$ versus LCV group.**

solution was added. The cells were transferred to transparent plate as 50 µl volume for per well and allowing detection of apoptosis by spectrophotometry at 550 nm (Synergy™ H1, Biotek, USA).

*Assay for caspase-3 and caspase-9 activities*

The determinations of caspase 3 and caspase 9 activities were based on methods previously reported (27, 28). MCF7 and Caco-2 cells were sonicated, and cell lysates were incubated with 2 ml of substrate solution (20 mM HEPES [pH 7.4], 2 mM EDTA, 0.1 % CHAPS, 5 mM DTT and 8.25 lM of caspase substrate) for 1 h at 37 °C. Caspase 3 substrate (NAcetyl-Asp-Glu-Val-Asp-7-amido-4-Methylcoumarin) (ACDEVD-AMC) and caspase 9 substrate (Ac-Leu-Glu-His-Asp-7-Amino-4-methylcoumarin) (AC-LEHD-AMC) cleavages were measured with the microplate reader (Synergy™ H1, Biotek, USA) with excitation wavelength of 360 nm and emission at 460 nm. The data were calculated as fluorescence units/mg protein and presented as fold-increase over the pre-treatment level (experimental/control).

*Mitochondrial membrane potential (JC-1) analyses*

The cells were incubated with JC-1 (1 µM concentration of 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimi-dazolylcarbocyanine iodide) at 37 °C for 45 min. JC-1 fluorescence was measured by a single excitation wavelength (488 nm) with dual emission, green (520 nm) and red (596 nm) using the microplate reader (Synergy™ H1, Biotek, USA). (29,30). The lipophilic cationic dye, JC-1, exhibits potential-dependent accumulation in mitochondria. It indicates mitochondrial depolarization by a decrease in the red-to-green fluorescence intensity ratio. After incubation with JC-1, the dye was removed, and the cells were washed in PBS. The green JC-1 signal was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm, and the red signal, at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were analysed using a fluorescence spectrophotometer (Synergy™ H1, Biotek, USA). Treatments were carried out in triplicate. Data are presented as emission ratios (590/535). Changes in mitochondrial membrane potential were quantified as the integral of the decrease in JC-1 fluorescence ratio of experimental/control.

*Cell viability (MTT) assay*

MTT Cell viability analysis was evaluated by the MTT assay based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. Cells were incubated with the chemical compounds described in the groups section. After treatments, the cells were washed and incubated with fresh Dulbecco's modified Eagle's medium (DMEM) containing MTT (0.5 mg/ml) at 37 °C for 90 min (31). Then, the supernatant was discarded and dimethyl sulfoxide was added to dissolve the formazan crystals. Optical density was measured in an automatic microplate reader (Synergy™ H1, Biotek, USA) at a test wavelength of 490 nm and a reference wavelength of 650 nm to nullify the effect of cell debris. The data are presented as fold-increase over the pre-treatment level (experimental/control).

*Statistical analyses*

All results were expressed as the means ± standard deviation (SD). Significant values in the groups were assessed with ANOVA and Mann–Whitney U test. Data were analysed using the SPSS statistical program (version 9.05 software, SPSS Inc. Chicago, Illinois, USA) $p < 0.05$ was considered significant.

## Results

*Effects of extracellular 5FU and LCV treatments on CMPx-induced ($Ca^{2+}$)i concentration through TRPM2 channels activation in the MCF7 and Caco-2 cells*

As the result of administration of 5-FU and LCV separately and together on breast cancer cell culture (MCF7) and colon cancer cell culture (Caco2), these agents increased the intracellular

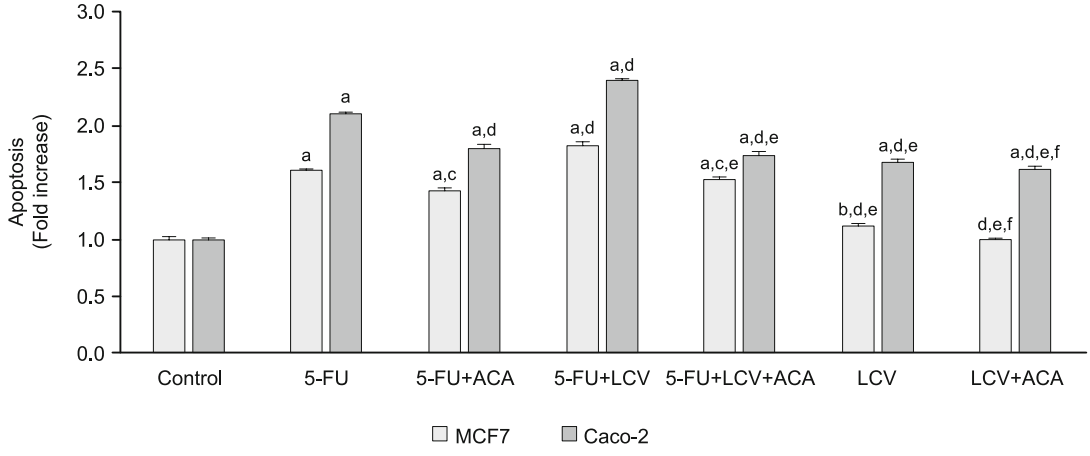


**Fig. 2. The effect of 5-FU (5 µM, 24 hrs) and Leucoverin (2 µM, 24 hrs) on apoptosis levels in the MCF7 and Caco2 cells. Cells are stimulated by Cumene hydroperoxide (CMPx 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (ACA 0.04 mM for 30 min) (mean ± SD and n = 10). [a]p < 0.001 and [b]p < 0.05 versus control group, [c]p < 0.05 and [d]p < 0.001 versus 5-FU group, [e]p < 0.001 versus 5-FU+LCV group and [f]p < 0.05 versus LCV group.**

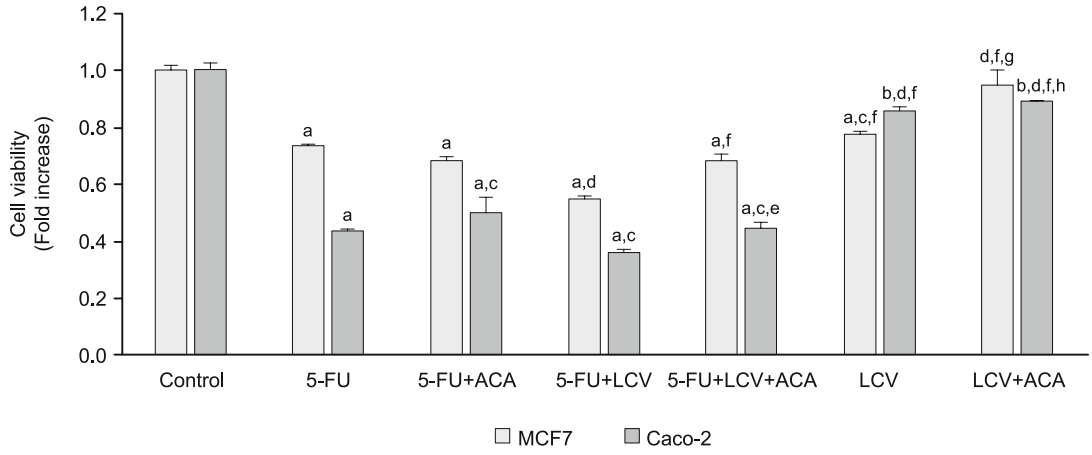


**Fig. 3. The effect of 5-FU (5 µM, 24 hrs) and Leucoverin (2 µM, 24 hrs) on Cell Viability levels in the MCF7 and Caco2 cells. Cells are stimulated by Cumene hydroperoxide (CMPx 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (ACA 0.04 mM for 30 min) (mean ± SD and n = 10). $^{a}p < 0.001$ and $^{b}p < 0.05$ versus control group, $^{c}p < 0.05$ and $^{d}p < 0.001$ versus 5-FU group, $^{e}p < 0.05$ and $^{f}p < 0.001$ versus 5-FU+LCV group, $^{g}p < 0.001$ and $^{h}p < 0.05$ versus LCV group.**

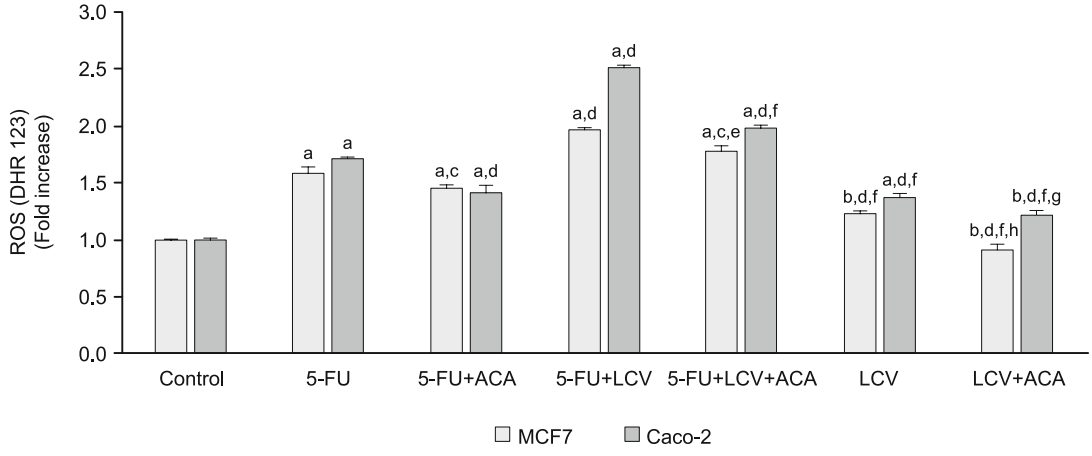


**Fig. 4. The effect of 5-FU (5 µM, 24 hrs) and Leucoverin (2 µM, 24 hrs) on Reactive Oxygene Species levels in the MCF7 and Caco2 cells. Cells are stimulated by Cumene hydroperoxide (CMPx 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (ACA 0.04 mM for 30 min) (mean ± SD and n = 10). $^{a}p < 0.001$ and $^{b}p < 0.05$ versus control group, $^{c}p < 0.05$ and $^{d}p < 0.001$ versus 5-FU group, $^{e}p < 0.05$ and $^{f}p < 0.001$ versus 5-FU+LCV group, $^{g}p < 0.05$ and $^{h}p < 0.001$ versus LCV group.**

calcium ($Ca^{2+}$) levels by stimulation of TRPM2 channels in both cancer cells statistically significantly compared to the control group. Intracellular ($Ca^{2+}$) levels were significantly increased ($p < 0.05$) by the use of TRPM2 channel stimulator (CMPx) in both cancer cells, especially in colon cancer, while using the TRPM2 channel inhibitor (ACA) reduced intracellular ($Ca^{2+}$) levels at a significant level ($p < 0.001$). Use of 5-FU and LV separately also showed these effects at a statistically significant level (stimulation: $p < 0.05$, inhibition: $p < 0.001$) (Fig. 1).

*Results of Apoptosis, MTT, ROS, Mitochondrial Depolarisation and Caspase 3 - Caspase 9 Values in MCF7 and Caco-2 Cells*

5-FU and LCV were administered separately and together to investigate the degree of apoptosis and cell viability. It was concluded that administration of TRPM2 channel stimulator (CMPx) in both cancer cells significantly increased a degree of apoptosis and decreased cell viability levels compared to the control group ($p < 0.001$), whereas the use of TRPM2 channel inhibitor (ACA), significantly decreased degree of apoptosis and increased cell viability levels ($p < 0.05$). It was found that this effect was statistically more significant in the 5-FU/LV combination group compared to the LV- only group ($p < 0.05$) (Figs 2 and 3).

As the result of investigation of mitochondrial depolarization levels and caspase 3 and caspase 9 levels after administration of 5-FU and LCV separately and in combination, it was found that Reactive Oxygen Species (ROS), mitochondrial depolarization and caspase 3 and caspase 9 levels in both cancer cells were statistically higher with the use of the TRPM2 channel stimulator (CMPx) compared to the control group ($p < 0.001$), whereas with use of the TRPM2 channel inhibitor (ACA), ROS and mitochon-

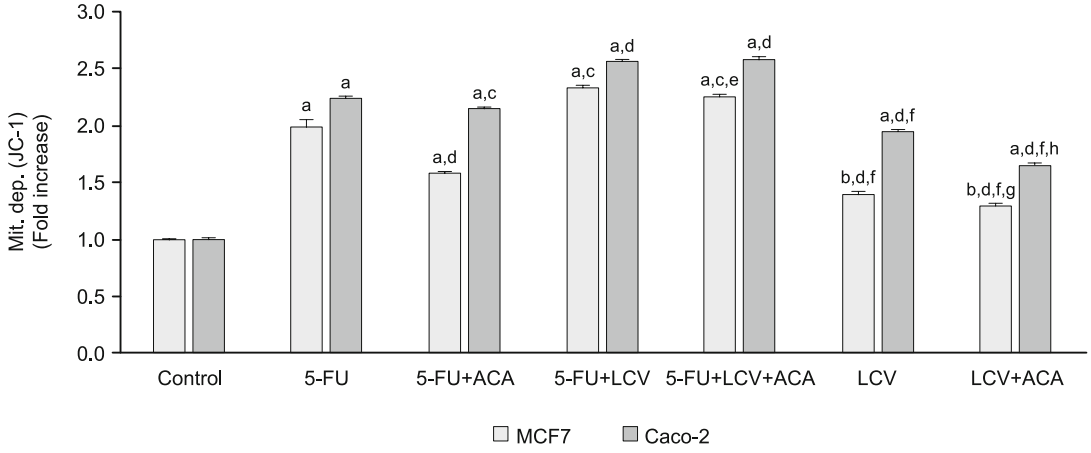


**Fig. 5. The effect of 5-FU (5 µM, 24 hrs) and Leucoverin (2 µM, 24 hrs) on Mitochondrial Depolarization levels in the MCF7 and Caco2 cells. Cells are stimulated by Cumene hydroperoxide (CMPx 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (ACA 0.04 mM for 30 min) (mean ± SD and n = 10). $^{a}p < 0.001$ and $^{b}p < 0.05$ versus control group, $^{c}p < 0.05$ and $^{d}p < 0.001$ versus 5-FU group, $^{e}p < 0.05$ and $^{f}p < 0.001$ versus 5-FU+LCV group, $^{g}p < 0.05$ and $^{h}p < 0.001$ versus LCV group.**

drial depolarization along with caspase 3 and caspase 9 levels were reduced significantly ($p < 0.05$). It was found that these effects were statistically more significant in the 5-FU/LV combination group compared to the LV-alone group ($p < 0.001$) (Figs 4, 5 and 6).

## Discussion

Breast and colon cancers are the most common cancers in the world and are among the leading causes of mortality. In the treatment of both cancers, various chemotherapy protocols are widely used in preoperative and postoperative periods. 5-FU chemotrapide, is widely used mainly in colon cancers, and is generally used in combination with 5-FU and LV (4).

TRP channels have 6 different subtypes in mammals; TRPA, TRPC, TRPM, TRPML, TRPP and TRPV. The selectivity of TRP channels has a wide spectrum, ranging from nonselective cation channels to highly selective ($Ca^{2+}$) channels. TRP channels respond to a variety of stimuli such as: ligands, heat, mechanical stimulation, and play a role in different processes such as intracellular ($Ca^{2+}$) metabolism, phagocytosis, cell motility, haemostasis, and inflammation (32–34). A number of studies revealed the effects of subpopulations of TRP channels on many different cancer cells and the relationship between the survival and the expression of TRP channels in these cancers have been clearly demonstrated (35).

Many recent studies showed an abnormal TRP channel expression in different types of cancer, and TRP channels have been reported to have effects on invasion, proliferation, differentiation, and tumour vascularization in cancer cells (36). TRPM2 channels have been reported to have abnormal expression and roles in many types of cancer, including adenocarcinoma, among which breast cancer is one of the most important malignancies. In one study, TRPM2 expression was significantly higher in in-situ breast cancer compared to the normal breast tissue, and TRPM2 expression in invasive breast cancer was reported to be 6-fold higher than in-situ cancer (15). The same study reported that TRPM2 could be used as a molecular biomarker for breast cancer invasion.

It is well known that intracellular ($Ca^{2+}$) levels have significant impact on cancer cells and TRP channels, and they have important roles in intracellular oxidative stress and apoptosis. It has been shown that TRP channels altered intracellular calcium concentrations and they had influence on regulation of ($Ca^{2+}$) release in numerous cell organelles (37). Intracellular ($Ca^{2+}$) concentration is variable and has been reported to increase in cases such as: increased proliferation, apoptosis and abnormal differentiation, which are indicative of cancer invasion (18,38). 5-FU, used therapeutically in many types of cancer, including breast and colon cancer, kills cancer cells by extremely increasing the amount of intracellular ROS (39). Increased intracellular ROS levels trigger oxidative stress, resulting in irreversible changes in components such as: intracellular lipids, proteins, and nucleic acids (40). The increase in intracellular ($Ca^{2+}$) levels leads to an increase in the amount of ROS, mitochondrial membrane depolarization and an increase in activation of caspase 3 and caspase 9 (23, 30, 41).

In our study, 5-FU and LV were administered separately and in combination on breast cancer cell culture (MCF7) and colon cancer cell culture (Caco2). Then the effects of these chemotherapeutic agents on TRPM2 channels and the effects of these channels on cell death were investigated. A specific stimulator (CMPx) and inhibitor (ACA) for TRPM2 channels were administered and the intracellular ($Ca^{2+}$) level, mitochondrial depolarization, caspase 3 and 9 values and intracellular ROS levels as well as degree of apoptosis were investigated by examining the intermediate stages of apoptosis of cancer cells and the results were compared to the control group. As the result of the analysis, it was found that the use of both drugs resulted in TRPM2 channel activation and that there was a significant increase in intracellular ($Ca^{2+}$) levels, mitochondrial depolarization levels and intracellular ROS levels mediated by TRPM2 channels in the 5-FU, 5-FU+LCV groups

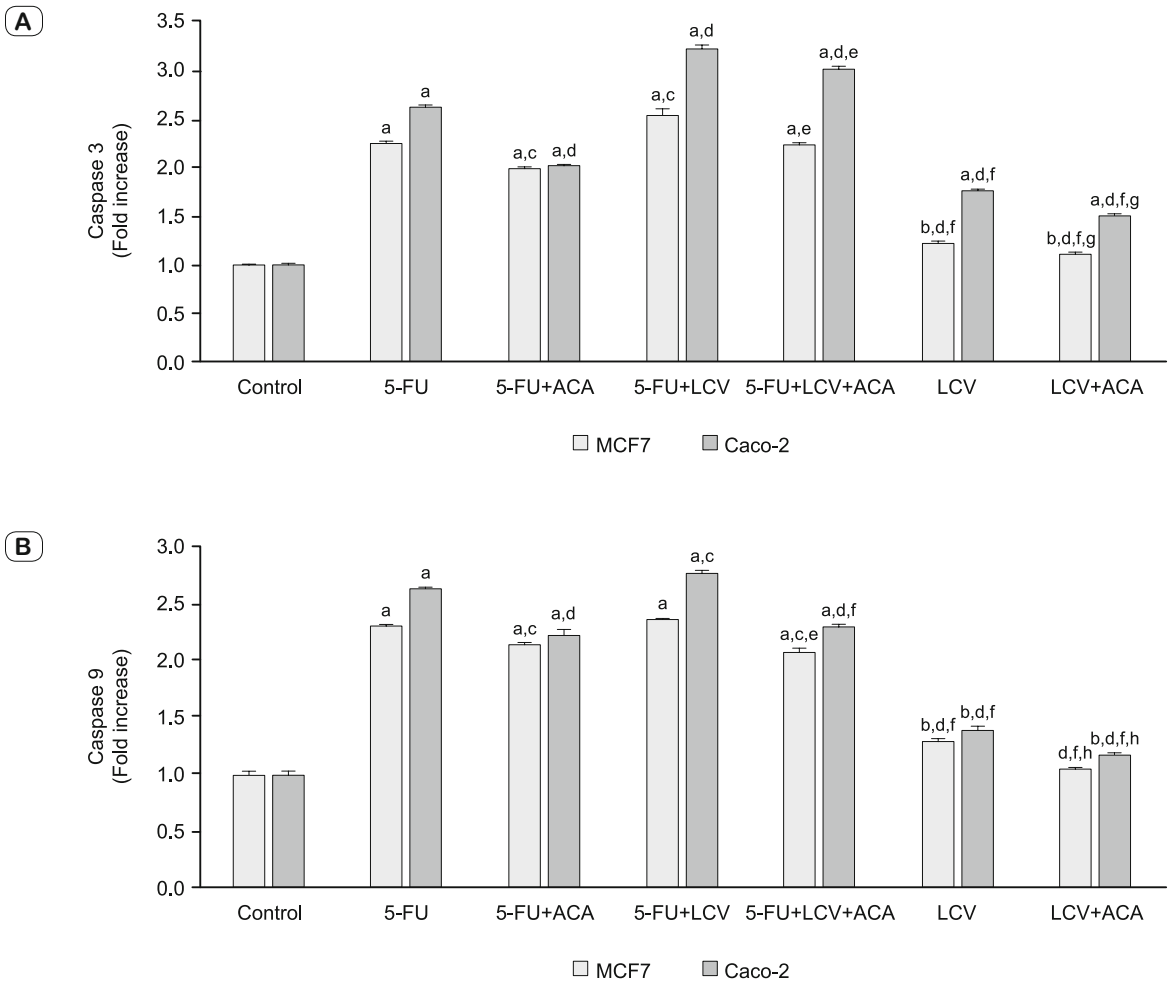


**Fig. 6. The effect of 5-FU (5 µM, 24 hrs) and Leucoverin (2 µM, 24 hrs) on Caspase 3 (A) and Caspase 9 (B) levels in the MCF7 and Caco2 cells. Cells are stimulated by Cumene hydroperoxide (CMPx 0.1 mM for 10 min) but they were inhibited by Antranilic Acid (ACA 0.04 mM for 30 min) (mean ± SD and n = 10). [a]p < 0.001 and [b]p < 0.05 versus control group, [c]p < 0.05 and [d]p < 0.001 versus 5-FU group, [e]p < 0.05 and [f]p < 0.001 versus 5-FU+LCV group, [g]p < 0.05 versus LCV group.**

and the oxidative stress-induced apoptosis levels were significantly increased compared to the control group. TRPM2 channel inhibition resulted in a statistically significant decrease in intracellular ($Ca^{2+}$) levels, mitochondrial depolarization levels and intracellular ROS levels and degree of apoptosis in the 5-FU+ACA, 5-FU+LCV+ACA groups compared to the groups without channel inhibition ($p < 0.05$). MCF7 and Caco2 cells showed a statistically significant increase in apoptosis levels ($p < 0.001$) in the 5-FU and 5-FU+LCV groups compared to the control group. Furthermore, the comparison of these effects of 5-FU and LV on both cancer types revealed that TRPM2 and all stages of cell death process were higher in colon cancer, being more pronounced in the 5-FU/LV combination. It was seen that 5-FU application alone had higher effects than LV alone ($p < 0.001$).

According to our literature review, there are very limited studies in literature on the effects of chemotherapeutic agents on TRP channels in colon and breast cancer, but no study has examined the effects of chemotherapeutic agents on TRPM2 channels in these cancers. In one study, TRPC5, another channel type that is active in oxidative stress in 5-FU chemoresistant colon cells, has been shown to play an important role (36). In another study regarding the effects of 5-FU on breast cancer cell culture on TRP channels and cell death, 5-FU was administered at a higher dose than our study (25 µM, 24 hrs). Similar to our study, where 5-FU was administered at a dose of 5 µM for 24 hours, they reported that 5-FU showed similar effect at all stages of the molecular pathway of apoptosis by increasing intracellular ($Ca^{2+}$) levels through TRPV1 channel activation (42). Increased TRPC5 expression in colon cancers was reported in literature (43), although TRPM2 channels were reported to play a role in tumour proliferation in breast cancer and may be used as invasion markers and play a role as prognostic factors (15). However, our study has established for the first time that TRPM2 channels had similar effects on colon cancer cells.

As the result of this study, we conclude that the apoptotic effects of 5-FU and LV, agents frequently used together for colon and breast cancer, were directly related to the TRPM2 channels and that these channels play an important role in all molecular pathways of apoptosis by elevating intracellular levels of ($Ca^{2+}$) and increasing mitochondrial depolarization. In addition, the results of our analyses showed that apoptotic activity of 5-FU+LCV combination in MCF7 and Caco2 cells involved TRPM2 channels and effect of 5-FU/LV on cell death through TRPM2 channels in colon cancer cells (Caco2) was higher than that of the breast cancer.

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