Research Article

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Effects of α-lactalbumin and sulindac on primary and metastatic human colon cancer cell lines

Sulindac ve α-laktalbuminin primer ve metastatik insan kolon kanseri hücre dizinleri üzerine etkileri

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Abstract

Background: HAMLET (human α-lactalbumin made lethal to tumor cells) and sulindac are active ingredients which are used in the treatment of cancers. Under both in vivo and in vitro conditions, HAMLET binds to the surface of tumor cells and enters the cells. Sulindac is one of nonsteroidal anti-inflammatory drugs. It inhibits the growth of tumor cells by inducing the apoptosis. In our study, we aimed to investigate effects of α-lactalbumin and sulindac on COLO-320 primary and COLO-741 metastatic human colon carcinoma cell lines via mitochondrial apoptotic pathway by indirect immunocytochemistry and TUNEL assays.
Materials and methods: The effects of α-lactalbumin and sulindac were assessed by using MTT assay at 24, 48 and 72 h and apoptosis markers caspase-3, caspase-9 and cytochrome-c were detected using immunocytochemistry and TUNEL methods.
Results: It was appeared that α-lactalbumin and sulindac may triggger mechanisms of apoptosis in both primary and metastatic colon carcinoma cell lines and the primary colon carcinoma cell line was affected more than the metastatic cells.
Conclusion: It is proposed that α-lactalbumin and sulindac can be used in cancer treatments and future in vivo experiments.

Keywords: Apoptosis; Cancer; In vitro; α-Lactalbumin; Sulindac.

Öz

Amaç: HAMLET (human α-lactalbumin made lethal to tumor cells) ve sulindac, kanser tedavisinde kullanılan etken maddelerdir. HAMLET, in vivo ve in vitro koşullar altında, tümör hücrelerinin yüzeyine bağlanarak hücre içerisine girer. Sulindac, non-steroidal bir anti-inflamatuvar ilaçlardan biridir ve apoptozu tetikleyerek tümör hücrelerinin çoğalmasını inhibe eder. Çalışmamızda α-laktalbumin ve sulindac'ın COLO-320 primer ve COLO-741 metastatik insan kolon karsinomu hücre dizinleri üzerindeki etkilerini, mitokondriyal apoptotik yolak aracılığı ile immünositokimya ve TUNEL yöntemleri ile araştırmayı amaçladık.
Gereç ve Yöntem: Sulindac ve α-laktalbuminin etkileri 24, 48 ve 72. saatlerde MTT metodu ile değerlendirildi ve immünositokimyasal olarak apoptoz belirteçleri kaspaz-3, kaspaz-9 ve sitokrom-c, ve TUNEL boyama yöntemleri uygulandı.
Bulgular: Sulindac ve α-laktalbuminin hem primer hem de metastatik kolon karsinoma hücrelerinde apoptoz mekanizmalarını tetikleyebileceği ve primer kolon karsinom hücrelerinin, metastatik hücrelerden daha fazla etkilendiği saptandı.
Sonuç: Sulindac ve α-laktalbuminin, gelecekte in vivo çalışmalarda ve kanser tedavilerinde kullanılabileceği düşünülmektedir.

Anahtar kelimeler: Apoptoz; Kanser; In vitro; α-Laktalbumin; Sulindac.

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Introduction

Colorectal cancer (CRC) is one of the causes of cancer-related deaths worldwide [1, 2]. The mortality rate of CRC hase rised considerably [3]. In the CRC treatment, surgery, chemotherapy or a combination of these procedures are used [4]. Chemotherapy is a substantial cure for CRC and alternative or consistent treatments are formed and tested. α-Lactalbumin and sulindac are active ingredients which have anti-tumorogenic effects [5–7].

α-Lactalbumin is the most abundant protein in female human milk [8]. It forms an active complex with oleic acid which is named HAMLET (human α-lactalbumin made lethal to tumor). Under both in vivo and in vitro conditions, HAMLET kills a variety of tumor cells such as human skin papilloma, colorectal and bladder cancer cells through the process of apoptosis, while healthy cells remain unaffected [5]. HAMLET translocates the cell nuclei by attaching to the surface of tumor cells, specifically interacts with histones, causing choromatin disruption and nuclear condensation [9]. Additionally, HAMLET initiates apoptosis by triggering the release of cytochrome-c from mitochondria [10].

Sulindac is one of the nonsteroidal anti-inflammatory drugs (NSAIDs) and it comes in two major metabolic forms, sulindac sulfide and sulindac sulfone [11]. It has been suggested that both sulindac metabolites have chemopreventive effects, causing apoptosis in numerous tumor cells such as colorectal cancer, human breast cancer cell line and human prostat cancer cell lines [12–15].

Apoptosis was originally described by Kerr et al. in 1972 [16]. It is the process of programmed cell death which is fundamental in embryonic development, organogenesis, morphogenesis, homeostasis and selection of immune cells [17]. Apoptosis can be characterized with chromatin condensation, tightly packed organelles and apoptotic bodies, which occurs through two major pathways (extrinsic and intrinsic pathways) depending on the source of death signals. Extrinsic or the death receptor pathway is initiated through the binding of trigger signal molecules such as FasL and tumor necrosis factor-α (TNF-α) to transmembrane receptors located in the cell membrane, whereas intrinsic or the mitochondrial pathway is activated by intracellular pro-apoptotic factors. Cytochrome-c is released from the mitochondria into the cytosol and binds with apoptotic protease activating factor-1 (Apaf-1) and adenosine triphosphate (ATP), this complex then binds to pro-caspase-9 to form an apoptosome which then cleaves the pro-caspase to its active form, caspase-9, allowing the caspase cascade to resume [17–19].

In the current study we aimed to examine apoptosis in α-lactalbumin and sulindac-treated COLO-320 primary and COLO-741 metastatic human colon carcinoma cell lines under in vitro conditions, in addition to their effects on mitochondrial pathways, using electron microscopy, TUNEL and indirect immunocytochemical assays.

Materials and methods

Cell lines and cell cultivation

The COLO-320 primary (HTL95027) and COLO-741 metastatic (HTL95008) human colon carcinoma cell lines were purchased from Interlab Cell Line Collection (Cenova, Italy) and were grown in RPMI-1640 (F1213, Biochrom, Berlin, Germany) medium containing 10% fetal bovine serum (FBS, S0113, Biochrom, Berlin, Germany), 1% L-glutamine (K0283, Biochrom, Berlin, Germany), and 1% penicillin/streptomycin (A2213, Biochrom, Berlin, Germany). The cell lines were maintained in a humidified atmosphere at 37°C in 5% CO_2. The medium was changed every other day and the cells passaged every 10 days. Cells were microscopically examined using an inverted microscope (IX71, Olympus, Japan) and photomicrographs were obtained.

Cytotoxicity assay

The cytotoxicities of α-lactalbumin (L6634, Sigma, St. Louis, MI, USA) and sulindac (S3131, Sigma, St. Louis, MI, USA) were measured using 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT, M5655, Sigma, Steinheim, Germany). COLO-320 and COLO-741 cells were passaged into 96-well (2.5×10^5 cells/well) plates (Corning, Darmstadt, Germany) and seeded for 24 h before drug treatment. After incubating, cells were treated with an increasing concentration of α-lactalbumin (0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM) and sulindac (0 μM, 1 μM, 10 μM, 100 μM, 1000 μM) for 24, 48 and 72 h. Drug applications were repeated every day due to the half-live of drugs being less than 24 h. Medium was then removed and 100 μL of fresh medium containing 10 μL MTT (5 mg/mL in distiled water) were added and incubated at 37°C for 4 h. MTT containing medium was then decanted and 100 μL of dimethyl sulphoxide (DMSO, A3672, AppliChem, Darmstadt, Germany) was added to each well, and absorbance was measured at a wavelength of 570 nm using an UV visible spectrophotometer multiplate reader (Versa Max,

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Molecular Device, USA) [20]. The 50% lethal doses (IC_{50}) of drugs were calculated using measurements of absorbance by GraphPad (GraphPad Software, San Diego, CA, USA).

Drug treatments

COLO-320 and COLO-741 cell lines were placed in 12-well plates at a density of 2.5×10^5 cells/per well and allowed to grow under routine conditions for 24 h. Both two types of tumor cells were randomly divided into four groups which were the control (no drug treatment), α-lactalbumin (IC_{50} dose, 0.250 μM), sulindac (IC_{50} dose, 18.75 μM) and a combination of both drugs. Cells were treated with drugs for 24, 48, 72 h. Drug applications were repeated every day due to the half-live of drugs being less than 24 h.

Transmission electron microscopy (TEM)

The cells were fixed in 2.5% buffered glutaraldehyde +2% paraformaldehyde in 0.1 M sodium phosphate buffer (16210, Electron Microscoby Sciences, Hatfield, PA, USA). The following day, cells were postfixed with 1% Osmium tetroxide (R1015, Agar Scientific, Essex, UK) at +4°C in the dark. After washing in buffer solution, cells were embedded into 2% agar (A 2114, AppliChem, Darmstadt, Germany) and were washed in buffer solution. Cells in agar were passed through 70% and 100% alcohol series (1 11727, Merk, Darmstadt, Germany) and were embedded into epon (2660, SPI-CHEM, West Chester, PA, USA) blocks and ultramicrotome (EM UC6, Leica, Vienna, Austria) was used for sections. The 50- to 100-nm thickness sections were examined with an EFTEM (Carl Zeiss Libra 120, Germany).

In Situ Cell Death Detection assay for apoptosis

Terminal deoxynucleotidyltransferase-biotin nick end-labelling (TUNEL) method was carried out to detect DNA fragmentation using a commercial in situ apoptosis detection kit (11 684 817 910, Roche, Mannheim, Germany). Cells were cultured in 12-well plates, after washing with phosphate buffered saline (PBS) (DMR E404-01, Diagnostic Biosystems, Pleasanton, CA, USA) twice and were incubated with 0.1% Triton X-100 (A4975, AppliChem, Darmstadt, Germany) for 15 min. The cells were again then rinsed with PBS twice. The samples were incubated with Terminal deoxynucleotidyl transferase (TdT)-enzyme for 1 h at 37°C in a humidified atmosphere. They were then washed with PBS three times and viewed using an IX71 inverted-florescence-phase microscope (Olympus, Japan) and photographed. As a negative staining control for TUNEL, TdT was omitted during the tailing of reactions. Staining was examined independently by the two authors, who both had no prior information regarding the samples. TUNEL positive cells were counted randomly from chosen fields (per sample) and data was presented as a percentage of the total amount of cells present. Cells in areas with necrosis or poor morphology were not analyzed [20].

Immunocytochemistry

After drug treatment, the medium was decanted and cells were rinsed with sterilized PBS and fixed with 4% paraformaldehyde in PBS at +4°C for 30 min. Following the fixation process they were washed with PBS three times for 5 min and permeabilized with 0.1% Triton X-100 (A4975, AppliChem, Darmstadt, Germany) in PBS at +4°C for 15 min. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide (H_2O_2, 1 08600, Merck, Darmstadt, Germany) for 5 min at room temperature. Cells were then washed with PBS and incubated with primary antibodies: anti- cytochrome-c (sc-13156, Santa Cruz, Heidelberg, Germany), anti-caspase 3 (C 5737, Sigma, St. Louis, MI, USA), and anti-caspase 9 (C 4356, Sigma, St. Louis, MI, USA) at +4°C overnight. After removal of the primary antibodies, cells were washed with PBS and incubated with biotinylated secondary antibodies and peroxidase-conjugated streptavidin using the protocol from a HISTOSTAIN kit (85-9043, Zymed, Carlsbad, CA, USA). Sample were then incubated with diaminobenzidine/hydrogen peoxide (K 3467, Dako-Cytomation, Carpinteria, CA, USA) and mounted with mounting medium (K002, DBS, Pleasanton, CA, USA). Stained samples were observed using a light microscope (IX71 inverted-florescence-phase microscope) (Olympus, Japan) and photographed [21]. The staining procedure was repeated three times and immunocytochemical staining was evaluated as weak (+), moderate (++) and strong (+++), respectively, in three different microscopic areas cells were counted for each intensity. The respective score was then calculated using the formula H-Score = Pi (intensity of staining +1). Pi is the percentage of stained cells for each intensity, varying from 0% to 100%. The H-score was evaluated by at least two investigators independently [22].

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Statistical analysis

The data was statistically expressed as mean ± SD values and were analyzed using repeated-measures ANOVA. The Tukey-Kramer multiple comparisons test was used to determine differences amongst the mean values. Values for $p \leq 0.05$ were considered significant.

Results

Cell viability and cytotoxicity

Cell viability and proliferation were determined using an MTT assay, in the presence of α-lactalbumin and sulindac. COLO-320 and COLO-741 cells were treated with α-lactalbumin at concentrations of 0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM and sulindac at concentrations ranging from 0 μM to 1000 μM for 24, 48 and 72 h (Figure 1). Starting at 10 μM, sulindac significantly increased cell death (***$p < 0.001$), and reaching 100% cell death at 1000 μM and at a IC_{50} dose of 18.75 μM. α-Lactalbumin caused 85% cell death at a concentrations of 10 and 100 μM (***$p < 0.001$) and its IC_{50} dose was 0.250 μM.

Transmission electron microscopy (TEM)

After treatment with α-lactalbumin, sulindac and/or a combination of the two drugs for 24, 48 and 72 h, transmission electron microscopic studies were conducted. The findings are presented in Figure 2. In the control group, COLO-320 and COLO-741 cells had normal nuclei and organelles which revealed a regular shape, integrated nuclear membrane and smooth surface. In the

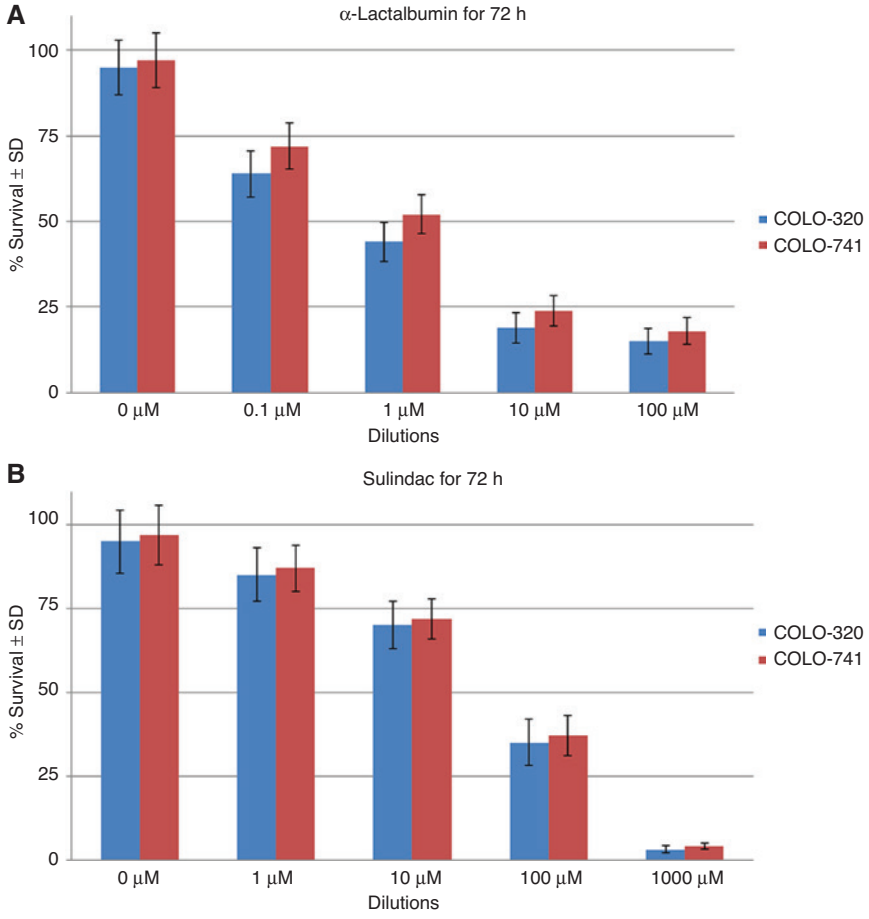


Figure 1: Survival of COLO-320 primary human colon carcinoma cells and COLO-741 metastatic human colon carcinoma cells exposed with α-lactalbumin (IC50: 0.250 μM) (A) and sulindac (IC50: 18.75 μM) (B) for 72 h was analyzed using MTT assay.
Percentage of cell survival was evaluated using One-way ANOVA with Tukey-Kramer Multiple Comparisons Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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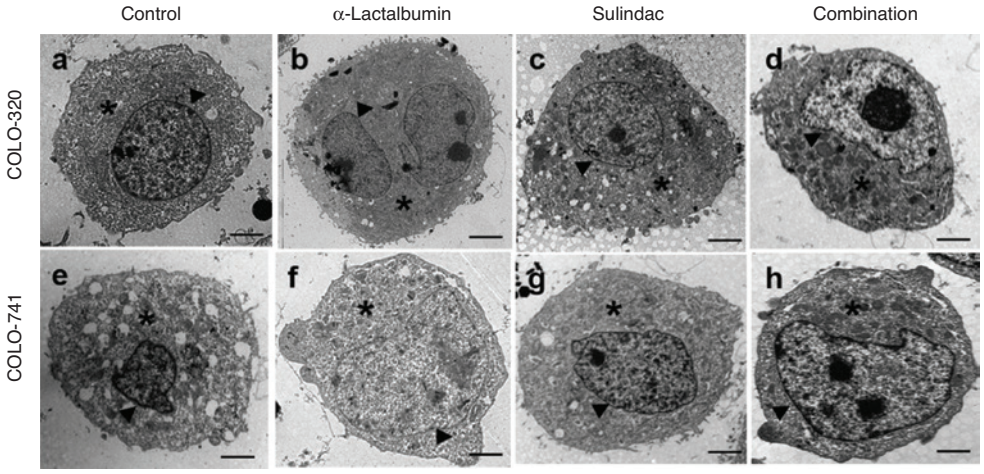


Figure 2: Findings of TEM studies.
Electron micrographs of COLO-320 primary human colon carcinoma cells (A: control, B: 0.250 μM α-lactalbumin, C: 18.75 μM sulindac, D: combination of IC50 doses) and COLO-741 metastatic human colon carcinoma cells (E: control, F: 0.250 μM α-lactalbumin, G: 18.75 μM sulindac, H: combination of IC50 doses) were exposed to the drugs for 72 h (arrow heads: nuclei, asterisk: cytoplasm). Scale bars: 0.2 μm.

case where treatment was with both drugs, chromosomal condensation and partial cell shrinkage were observed. α-Lactalbumin and sulindac exposed COLO-320 cells exhibited more cell damage compared with COLO-741 cells. There were no significant differences between 24, 48 and 72 h applications of α-lactalbumin and sulindac in both cell lines.

In Situ Cell Death Detection and immunocytochemistry assay

The apoptotic effects of α-lactalbumin and sulindac on COLO-320 and COLO-741 cells and distribution of caspase-3, caspase-9 and cytochrome-c were observed using immunocytochemistry and TUNEL assays.

The percentage of apoptotic cells is presented in Figure 3. The number of TUNEL positive cells increased in all treated groups especially in the COLO-320 cell line compared to COLO-741 cells. α-Lactalbumin and sulindac caused cell death of COLO-320 after application for 24, 48 and 72 h compared to control group (***$p < 0.001$) (Figures 3 and 4). The number of TUNEL positive cells in the combination group were at 24, 48 and 72 h were greater than the control (***$p < 0.001$), α-lactalbumin and sulindac groups (**$p < 0.01$). The difference between treatments with α-lactalbumin and sulindac were not statistically significant ($p > 0.05$). In the group of COLO-741 cells, apoptotic cells increased following the administration of the two drugs independently and in combination, whereas apoptotic cells were observed rarely in the control group (***$p < 0.001$). The increase of apoptotic cells in the combination group was statistically significant (**$p < 0.01$) (Figure 3). The combination of the IC_{50} doses of two drugs had the most toxic effect on COLO-320 cells (Figures 4 and 5).

After investigation of immunocytochemical stainings, the localization of caspase-3, caspase-9 and cytochrome-c were identified in the cytoplasm, especially caspase-3 was located near the nucleus. Immunoreactivities of caspase-3, caspase-9 and cytochrome-c were detected in all treated groups, these immunoreactivities increased in groups which were treated with both drugs compared with the control groups. There were no statistically significant differences between 24, 48 and 72 h applications ($p > 0.05$) (Figure 6). The caspase-3 stained cells were rare in the control groups of both two cell lines. The caspase-3 immunoreactivity increased following the combined application of the IC50 doses of two drugs on COLO-320 cells (***$p < 0.001$) (Figure 6A). The number of cytochrome-c showing cyptoplasmic staining of cells increased in the α-lactalbumin and sulindac treated COLO-320 cells compared with control group (***$p < 0.001$) and also caspase-9 stained cells were enhanced in the treated groups (***$p < 0.001$). In the COLO-741 cells, combination of IC50 doses of α-lactalbumin and sulindac administration caused a significant increase in the immunoreactivity of cytochrome-c when compared with the control group (***$p < 0.001$) (Figure 6F). The immunoreactivities of caspase-3 and caspase-9 were similar in all treated groups of COLO-741 cells and significant difference was not found between the α-lactalbumin and sulindac applications ($p > 0.05$). Immunoreactivities of caspase-3, caspase-9

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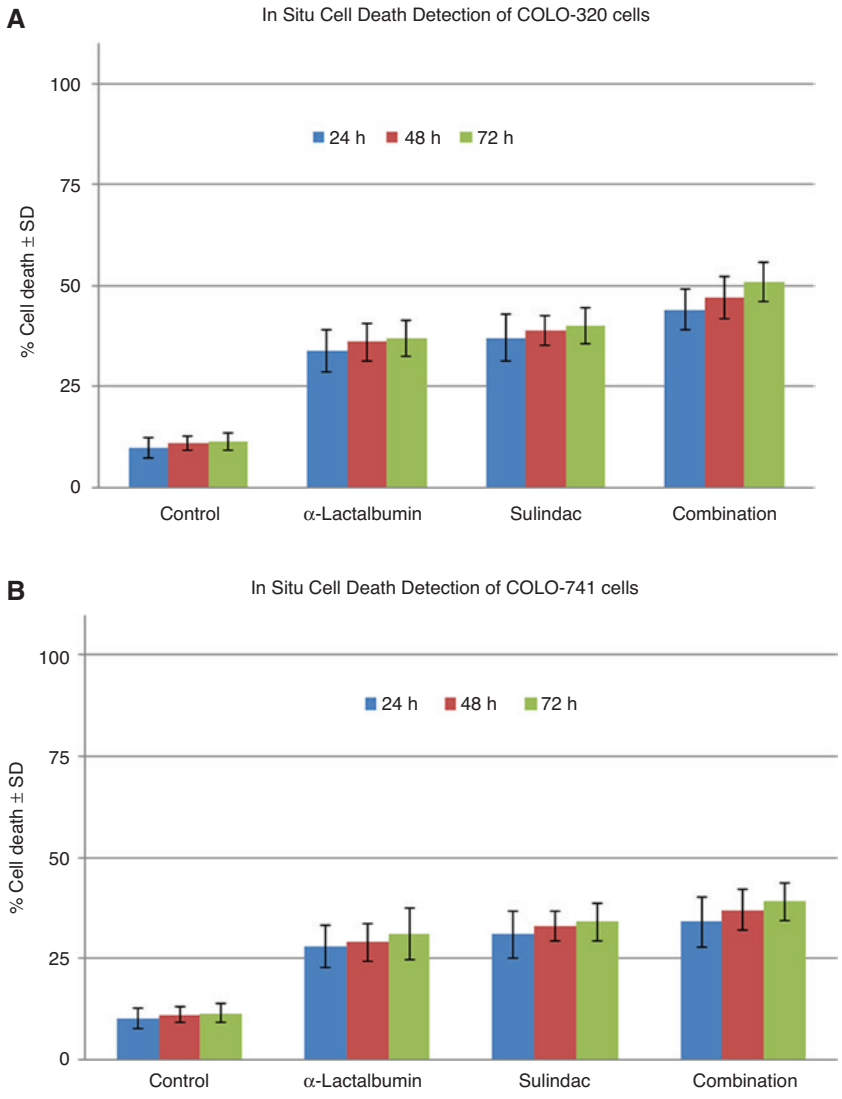


Figure 3: In Situ Cell Death Detection of COLO-320 primary human colon carcinoma cells (A) and COLO-741 metastatic human colon carcinoma cells (B) after applications of α-lactalbumin (0.250 μM), sulindac (18.75 μM) and combination of the IC50 doses of two drugs for 24, 48 and 72 h.
Percentage of apoptotic cells was evaluated for each drug (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

and cytochrome-c were the greatest in the treated groups of COLO-320 cells in comparison to the COLO-741 cells (Figure 6D, E).

α-Lactalbumin and sulindac had significantly more toxic effects on COLO-320 cells than on COLO-741 cells. We found a positive relationship between the number of apoptotic cells and immunoreactivities of caspase-3, caspase-9 and cytochrome-c.

Discussion

In the current study, we demonstrated that COLO-320 primary human colon carcinoma cells and COLO-741 metastatic human colon carcinoma cells were killed by sulindac and α-lactalbumin treatment and moreover treatment using a combination of these drugs had more of an effect on these cell type. According to results of apoptosis assay, using combination of IC_{50} doses of these drugs was more toxic on the both colon cancer cells. Hovewer, metastatic cells were more resistant to these factors when compared with that of primary cells. We were also able to demonstrate that the effects of these drugs originated from an apoptotic pathway which was shown by cytochrome-c, caspase 3, caspase 9 immunocytochemically, TUNEL and electron microscopy. These factors for apoptosis were more pronounced by primary cells compared to that of metastatic cells. The results have

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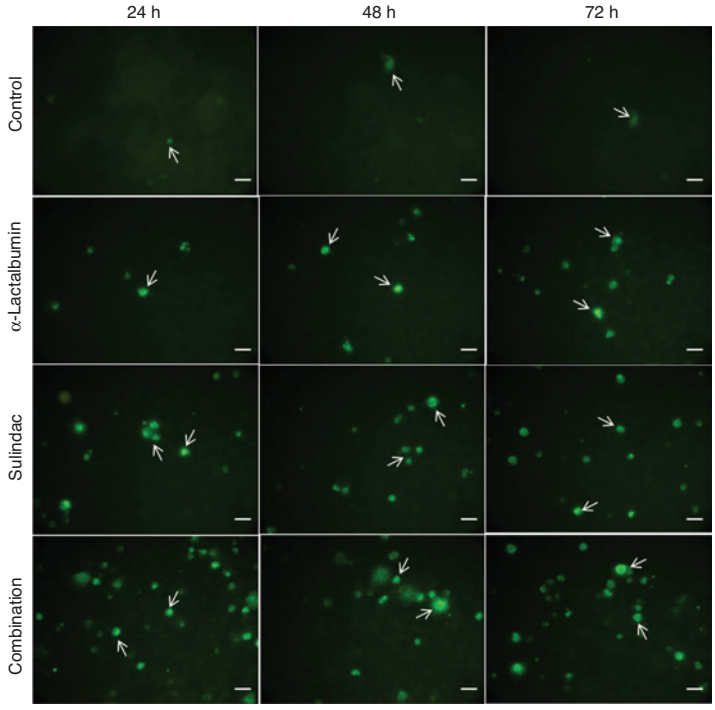


Figure 4: In Situ Cell Death Detection of COLO-320 primary human colon carcinoma cells after application with α-lactalbumin (0.250 μM), sulindac (18.75 μM) and the combination of of IC50 doses of both drugs.
Arrows: Apoptotic cells. Scale bars: 20 μm.

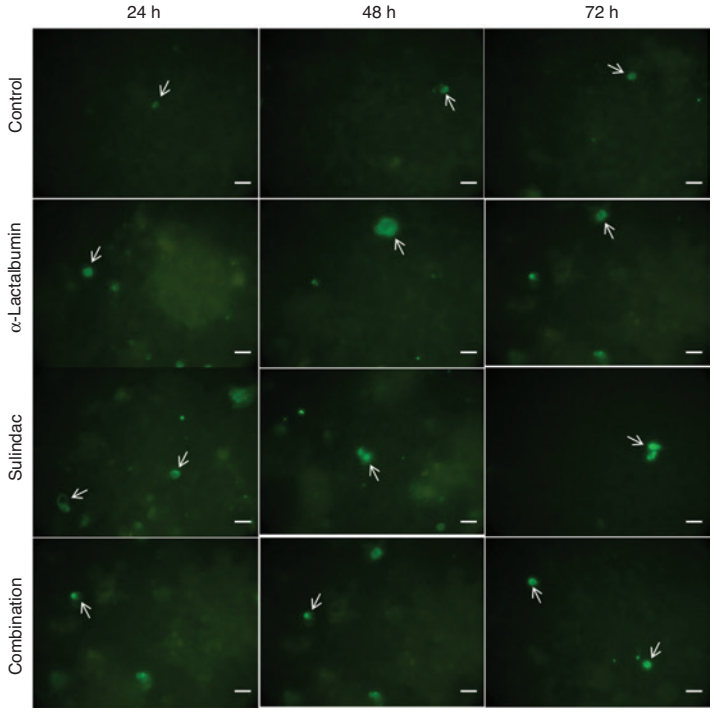


Figure 5: In Situ Cell Death Detection of COLO-741 metastatic human colon carcinoma cells after application of α-lactalbumin (0.250 μM), sulindac (18.75 μM) and the combination of of IC50 doses of both drugs.
Arrows: Apoptotic cells. Scale bars: 20 μm.

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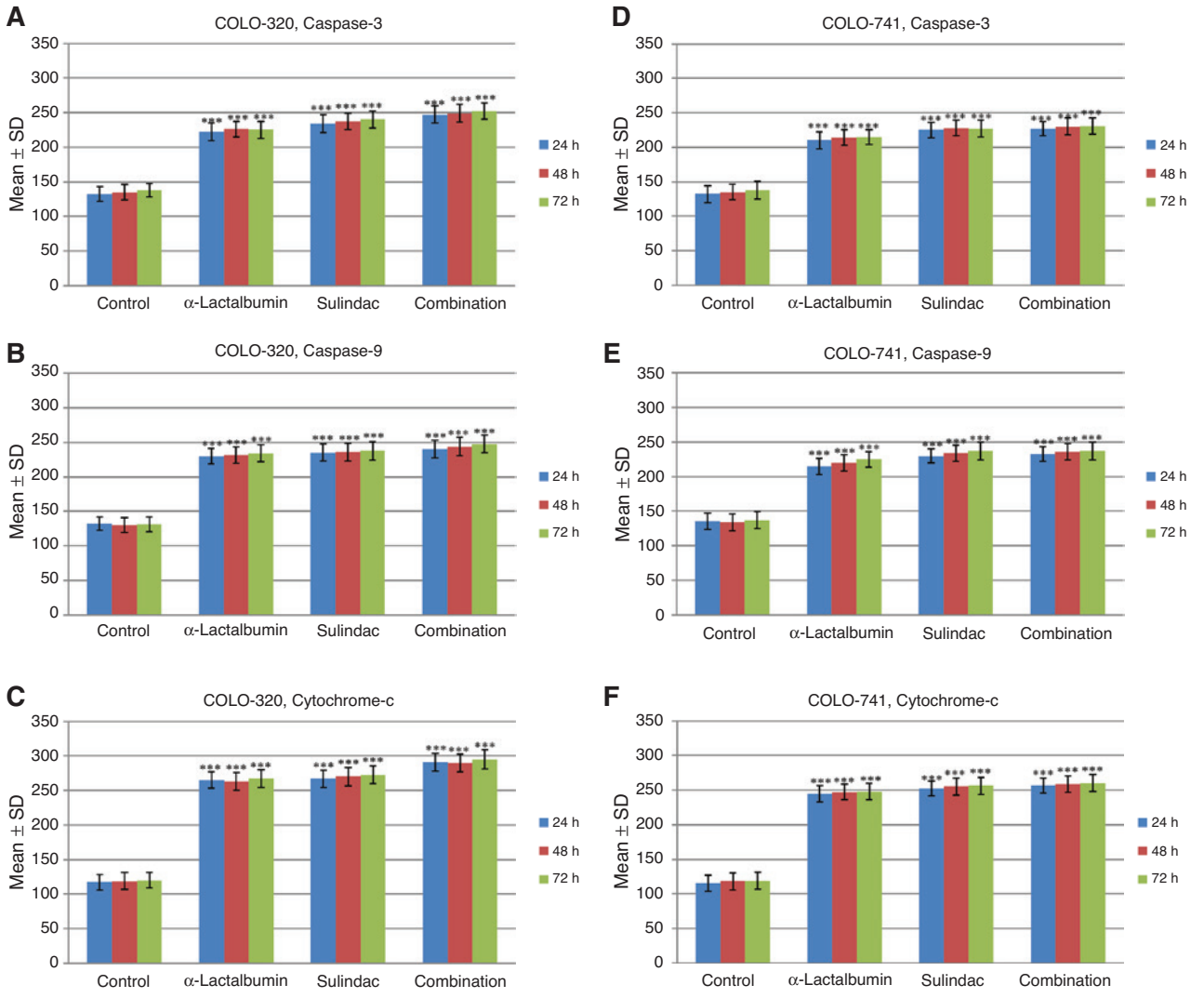


Figure 6: The H-Score analysis of immunocytochemical staining of caspase-3, caspase-9 and cytochrome-c in COLO-320 primary human colon carcinoma cells (A–C) and and COLO-741 metastatic human colon carcinoma cells (D–F) after application with α-lactalbumin (0.250 μM), sulindac (18.75 μM) and the combination of of IC50 doses of both drugs for 24, 48 and 72 h (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

supported our claim that apoptosis occurred via mitochondrial pathway in our experiment. The data clearly suggest that the combined use of both drugs can be used for colon cancer treatment.

Sulindac is a non-steroidal anti-inflammatory drug. Previous studies shown that sulindac triggered apoptosis in human colon cancer cells. In HT-29 colon cancer cells, sulindac caused decrease of β-catenin levels and initiation of apoptotic cell death. The inhibition of Wnt/β-catenin signaling prevented the survival of HT-29 colon cancer cells [23]. Sulindac was observed to trigger apoptosis in HCT-15 colon cancer cells. HCT-15 cell death increased by the activation of NF-Kb [12]. In SW480 human colon cancers cells studied under in vitro conditions, apoptosis detection was via death receptor 5 (DR5). SW480 cells were treated for 24 h with sulindac at a concentration of 200 μM, 10 ng/mL TRAIL, 20 μM zVAD-fmk and also in the combined presence of these factors. Western blotting analysis revealed an increase in the levels of caspase-8, caspase-3 and PARP. Sulindac was administrated to the SW4820 cells in concentrations of 0, 25, 50, 100 and 200 μM to inhibit proliferation of cancer cells. The expression of DR5 was upregulated following the treatment with sulindac at a concentration of 200 μM. It became evident that sulindac induced DR5 and caspase protein activity such as caspase-8 and caused cell death via apoptosis [6]. Previous experiments indicated that anti-tumorogenic effects of sulindac were in a dose-dependent manner. Similarly, the current data showed that sulindac caused cell death by increased immunostainings of caspase-3,

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caspase-9 and cytochrome-c were found in the cytoplasm with the application of sulindac. Thus, it can be used effectively on colon cancer cell lines.

HAMLET consists of Ca^{+2} binding protein α-lactalbumin, which can be obtained from female human milk, and fatty acid (oleic acid) [8]. In tumor cells, HAMLET damages the chromatin irreversibly, and activates the caspases by effecting the mitochondria and inducing apoptosis [7, 9, 10, 24]. The anti-tumorogenic effect of HAMLET has been detected using a variety of tumor cells such as human skin papilloma, colorectal and bladder cancer, breast and lung cancer cell lines, glioblastoma by apoptosis, but not healthy cells or non-transformed cells [5, 25]. In vivo experiments show that HAMLET inhibits the proliferation of tumor cells while under in vitro conditions it triggers cell death in the different types of cancer cells (A549, HT-29, SK-BR-3, DU145, Jurkat cell lines). The Jurkat cells were exposed to 18 μM of HAMLET and during cell death, apoptotic signs were ascertained such as caspase activation, phosphatidyl serine release and chromatin condensation, but the response of caspase was independent from the Bcl-2 [26]. It was stated that HAMLET has an inhibitory effect on tumor progression in Apc(Min)(/+) mice. Apc(Min)(/+) mice were administrated with HAMLET (10 mg/day) perorally for 10 weeks and there was a reduction in the size and number of intestinal tumors compared with the control group. HAMLET was shown to diminish the tumor size but not affect the healthy tissue, also expressions of β-catenin, cyclin D1, COX-2 and VEGF in intestinal tumor tissue were significantly reduced in the HAMLET treated group. It was suggested that HAMLET killed tumor cells via Wnt/β-catenin signaling pathway [5]. In our study we suggest that α-lactalbumin killed the colon cancer cells via the mitochondrial apoptosis pathway. We also investigated the ultrastructure of the colon cancer cells in culture to assess the effects of drug treatment. There were apoptotic signs amongst both primary and metastatic cells but it was particularly more pronounced in the primary cells. Electron microscopy images of cancer cells showed that the addition of sulindac and α-lactalbumin induced increased chromatin condensation and partial cell shrinkage. These results strongly suggested that sulindac and HAMLET use have similar effects on the colon cancer cells as has been shown in previous studies.

Our results especially suggest that the behavior of primary tumor cells may be different from metastatic cells where we should produce more effective treatment to cure metastatic colon cancer. In accordance with our results sulindac and α-lactalbumin should used together on colon cancer cells in order to decrease proliferation and increase apoptosis. Further activation of apoptotic factors such as cytochrome-c, caspase-3 and 9 and TUNEL demonstrated that these are the molecular mechanisms of drug induced apoptosis in primary and metastatic colon cancer cells. These mechanisms should be further investigated in order to improve the effectiveness of potential chemopreventive agents against colon cancer.

Limitation of this study

In this study, we aimed to investigate the activity of both substances in IC_{50} doses. We investigated their IC_{50} dose activity separately and compared them with combinations of these doses. The combinations of different concentrations were not evaluated. Some figures have less clarity for interpretation.

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Declaration of interests

The authors declare no conflict of interests.

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