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Investigation of Methylation Profiles of TP53, Caspase 9, Caspase 8, Caspase 3 Genes Treated with DNA Methyl Transferase Inhibitor (DNMTi) Zebularine (ZEB) and Caffeic Acid Phenethyl Ester (CAPE) on MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

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Abstract

Purpose: MCF-7 (ER+, WTP53) and MDA-MB-231 (ER Met, Mutant P53) Caffeic Acid Phenethyl Ester (CAPE) and DNA Methyl Transferase Inhibitor (DNMTi) in breast cancer cell lines of Zebularine (ZEB) single and combined application of TP53, caspase-9, caspase 8 and caspase-3 genes as a result of the use of single and combined drug methylation profiles are aimed to be evaluated by specific PCR method. **Material-Metods:** In the MCF-7 and MDA-MB-231 breast cancer cell lines, MTT test and survival analysis were performed as a result of single and combined application of CAPE and Zebularine and Methylation Specific PCR was performed to examine the methylation of caspase-3, caspase-8, caspase-9 and TP53 genes. **Results:** According to the results of 24-hour drug administration, the IC_{50} for the MCF-7 cell line was determined as 200 μM, for CAPE 40 μM and for the combined values of 50 μM ZEB + 5 μM CAPE. The effects of caspase-3, caspase-8, caspase-9 and TP53 genes on the methylation level of ZEB, CAPE and ZEB + CAPE drug combination were determined by using bisulfite modified DNAs in MCF-7 and MDA-MB-231 cell lines. **Discussion:** In the MCF-7 cell line, the 120 μM ZEB viability rate was 51%, and the viability of

80 μM ZEB MDA-MB-231 breast cancer cells decreased by 59.7%. After 20 μM CAPE, viability in MCF-7 cells decreased by 31% in 120 μM CAPE and MDA-MB-231 cells decreased by 41%. The viability with 40 μM CAPE decreased by 19% in MDA-MB-231 cells. It was found that 20 μM CAPE concentration was associated with TP53 methylation in MCF-7 cell lines. The 80 μM ZEB concentration was found to be closely related to the unmethylated status of the TP53 gene. These results obtained with 50 μM ZEB + 5 μM CAPE application were found to be related to the methylated-unmetylated status of the TP53 gene in half (50%). For the caspase-9 gene of MDA-MB-231 cells, 80 μM ZEB concentration was found to be associated with unmetylated status. The effective use of drugs with low concentrations of the drug dose provides a more appropriate approach in terms of treatment.

Keywords

MCF-7, MDA-MB-231, Zebularine, CAPE, Breast Cancer, Methylation

1. Introduction

Breast cancer is a common type of cancer and, despite improvements in its treatment, it is still considered to be the main cause of death in women [1]. Studies have shown that breast cancer is a heterogeneous tumor that responds differently to treatments. Breast cancer is caused by the accumulation of genetic and epigenetic events. Most of the epigenetic modifications are reversible events that target the gene sequence after transcription and the inhibition of these mechanisms may be advantageous in the treatment of breast cancer [2]. DNA methylation is an important epigenetic modification that is most studied and involved in the regulation of many cell processes beyond the DNA sequence level [3]. Cancer cells often exhibit abnormally high DNA methylation at gene-specific CpG-rich promoter sites [4]. DNA methylation is controlled by DNA methyl transferase (DNMT), an enzyme that catalyses the transfer of a methyl group from S-adenosyl-1-methionine to the 5-position of cytosines in the CpG nucleotide. DNMT overexpression was detected in various malignancies, including lung, prostate, and colorectal tumors [3] [4]. Since DNA methylation is a reversible biochemical process, and DNMT inhibitors are not specific to cancer type, DNMT may be a viable target in cancer treatment, since it can be used in the treatment of various cancers [5]. By inhibiting DNMTs, genes that may be silenced by DNA methylation during the carcinogenic process can be reactivated and the non-carcinogenic state of the cell can be regenerated [6]. Because of this feature, DNA methylation changes are believed to be an alternative pathway for cancer and have the potential to be diagnostic markers that can be used in the clinical context [7].

Zebularine (ZEB), a novel DNMT inhibitor, is a sitidine analogue [8] [9]

containing the 2-(1H)-pyrimidinone ring initially developed as a cytidine deaminase inhibitor to prevent deamination of nucleoside analogs. Zebularine is a versatile starting material for the synthesis of complex nucleosides and is a mechanism based on mechanism DNA cytosine methyl transferase inhibitor [10]. The mechanism of action of ZEB as a DNMTi is its incorporation into DNA after its phosphorylation to the diphosphate level and its conversion to a deoxynucleotide. Unlike other DNMTi, zebularine is less toxic for breast cancer cell lines and there are antimitogenic and angiostatic effects of ZEB. It has been shown that ZEB has antitumor activity by stimulating apoptosis and has an effect of inhibiting cell proliferation [2]. Caffeic acid phenethyl ester (CAPE) is polyphenol, a component of honeybee propolis. CAPE has a number of important biological activities including anti-bacterial, antiviral, antifungal, antioxidant, anti-inflammatory and anticancer properties [11]. CAPE has been supported by studies on cell cycle progression, cell proliferation, cell cycle arrest and apoptosis in many cancer models (colon, lung, pancreatic).

CAPE inhibits the activity of cancer cells using nuclear transcription factor NF-kB. Studies have shown that NF-kB may be one of the most important factors in oncogenesis and cancer progression. In addition, CAPE is capable of exhibiting different toxicity to tumor models without affecting normal cells [12]. Drug metabolism times of those two kinds of DNA methyl transferase inhibitors CAPE and ZEB are 24 to 96 hours.

Apoptosis (programmed cell death) is a critical obstacle to tumorigenesis. Proteins such as calcium, ceramide, Bcl-2 family, P53, caspases, cytochrome-c and mitochondria serve in the regulation of apoptosis. Caspases are a cysteine protease family and function as central regulators of cell death. TP53 is mutated in most cancers and mutated in 30% - 50% of diagnosed breast tumors.

Chemotherapeutic agents may affect many effector and regulatory elements of the process of apoptosis. Several studies have shown that chemopreventive agents induce apoptosis in various cancer cells by affecting multiple proteins involved in programmed cell death. In accordance with the need for new compounds with both effective antitumor effect and high cancer cell selectivity and low normal cell toxicity, recently some compounds have increased the need for natural compounds to inhibit cancer cell growth [13].

In the first stage of this study, the DNA metabolite transferase inhibitor Zebularine is a new generation drug with propolis extract Caffeic Acid Phenethyl Ester (CAPE)'s MCF-7 breast cancer cell lines first and the combined use of drugs as a result of IC_{50} values The aim of this study was to determine the effects of ZEB, CAPE and ZEB + CAPE on cell viability, cell proliferation and to determine the relationship between epigenetic and caspases known to be associated with apoptotic pathway. Although different applications of ZEB and CAPE drugs in different breast cancer cell lines exist in the literature, the combined use of these two drugs is not observed. Therefore, the original value of this study is preserved.

2. Materıals and Method

2.1. Cell Culture

MDA-MB-231 (ER (-), Mutant P53) and MCF-7 (ER (+), WTP53) human breast cancer cell lines 10% heat inactivated fetal bovine serum (FBS), 2 mM L gulutamine, 100 µg/ml were cultured in RPMI 1640 medium, containing penicilin-streptomycin, at 37˚C and 5% CO_2 incubator. (Memmert CO_2 Incubator, INCO153med, Germany).

2.2. Cell Viability Test (MTT Test)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test is performed on incubated breast cancer cell lines to determine the time-dependent effects of ZEB and CAPE in different concentrations. Cells are treated with 0 - 200 µM ZEB and 0 - 40 µM CAPE for 24 hours. At 570 nm wavelength spectrophotometric reading, the effect of drugs on relative cell viability was determined by controlling and proportioning to the drug applied cells. (Multiskan™ FC Microplate Photometer, ThermoFisher Scientific, Multiskan FC, China.)

2.3. Survival/Trypan Blue Coloring Test

The aim of this study is to determine the time-dependent effect of the drugs administered with survival test at a certain concentration. For this purpose, the MDA-MB-231 and MCF-7 breast cancer cell lines are seeded in 6-wells and the cells are treated with drugs at 24, 48, 72 and 96 hours according to the IC_{50} values obtained for the drugs. Counting of cells was performed on Neubauer hemocytometer.

2.4. Determination of the Effects of Applied Drugs on Epigenetics

2.4.1. DNA Isolation

Total DNA isolation from breast cancer cells was performed to determine the effect of applied drugs on methylation and total DNA concentrations isolated were measured in nanodrop device. (Nanodrop, Shimadzu Corporation, BioSpec-nano, Japan).

2.4.2. Bisulfite Modification and Methylation Specific PCR (MSP)

It is the process of converting unmetylated cytosine residues to uracil without any change in methylated cytosines. Zymo EZ DNA Methylated-GoldTM kit (D5005, Zymo Research Corp., Orange, CA) was used for the bisulfite conversion of genomic DNA. The bisulfite transformed DNA was used as a template and MSP method was applied to determine the methylation status of the genes that obtained significant results, especially caspase 3, caspase 8 and caspase 9 (Table 1). (Thermal Cycler Block, ThermoFisher Scientific, 5020, Finland.) PCR reaction 95˚C 5 min initial denaturation, 40 cycles; 94 - 30 s, 50˚C - 30 s, 72˚C 30 s and finish 72˚C 7 min and the PCR results were examined on the non-denaturing gel.

Table 1. The primers designed for methylated (M) and non methylated (UM) areas in -PCR reaction (F: Forward, R: Reverse).

Genes	Primers
Caspase 8	M-F: 5'-TGTTGTTTGGGTAACGTATCGA-3' M-R: 5'-CCCTACTTAACTTAACCCTACTCGAC-3' U-F: 5'-TTGTTGTTTGGGTAATGTATTGA-3 U-R: 5'-CAACCCTACTTAACTTAACCCTACTCA-3
Caspase 3	M-F: 5'-TTT AGG GCG GGA TTA AAG C-3' M-R: 5'-CTACGACCCGTCCCCTAA-3' U-F: 5'-TGAGTTTTAGGGTGGGATTAAAGT-3' U-R: 5'-TTCGGTAGGCGGATTATTTG3'
Caspase 9	M-F: 5'-GGGAGC GAAGATTGATTC-3 M-R: 5'-CTTCGTCCATAACGAATAACC-3 U-F: 5'-GTGGGGAGTGAAGATTGATTT-3' U-R: 5'-CCACTTCATCCATAACAAATAACC-3
P53	M-F: 5'-TTCGGTAGGCGGATTATTTG3' M-R: 5'AAATATCCCCGAAACCCAAC-3' U-F: 5'-TTGGTAGGTGGATTATTTGTTT-3' U-R: 5'-CCAATCCAAAAAAACATATCAC-3'

3. Results

3.1. The Effect of Combined Application of ZEB, CAPE and ZEB + CAPE on Cell Viability in MCF-7 and MDA-MB-231 Breast Cancer Cell Lines

In order to determine the appropriate dose of Zebularine MCF-7 and MDA-MB-231 breast cancer cells, changes in cell viability after 24 hours of drug application at increasing dose concentrations of 0 - 200 μM were examined by MTT cell viability test (**Figure 1(a)**). It was observed that the vitality rate in MCF-7 breast cancer cells after 24 hours of administration with 200 μM ZEB was 51% (**Figure 1(a)**) and the viability of MDA-MB-231 breast cancer cells decreased by 59.7% after 24 hours of administration with 80 - 200 μM ZEB. (**Figure 1(b)**) ($p < 0.05$).

In order to determine the appropriate dose of CAPE on MCF-7 and MDA-MB-231 breast cancer cells, changes in cell viability were determined by MTT cell viability test as a result of drug administration at an increasing dose range of 0 - 40 μM for 24 hours. After 24 hours of administration with 20 μM CAPE, viability in MCF-7 cells decreased by 31% (**Figure 2(a)**), and after 24 hours of administration with 120 μM CAPE, viability in MDA-MB-231 cells decreased by 41%. Similarly, viability with 40 μM CAPE decreased by 19% in MDA-MB-231 cells. (**Figure 2(b)**) ($p < 0.05$).

To determine the effective combination doses of ZEB and CAPE on MCF-7 and MDA-MB-231 breast cancer cells, over 1/4 of the doses determined by the MTT test using ZEB and CAPE alone and supported by the survival test combined drug range applied. In the MCF-7 breast cancer cells, ZEB: 50 μM + CAPE: 5 μM combined dose decreased by 50% after 24 hours of administration

(Figure 3(a)). In MDA-MB-231 breast cancer cells, ZEB: 20 μM + CAPE: 30 μM combined dose decreased by 41% after 24 hours of administration. (Figure 3(b)) ($p < 0.05$).

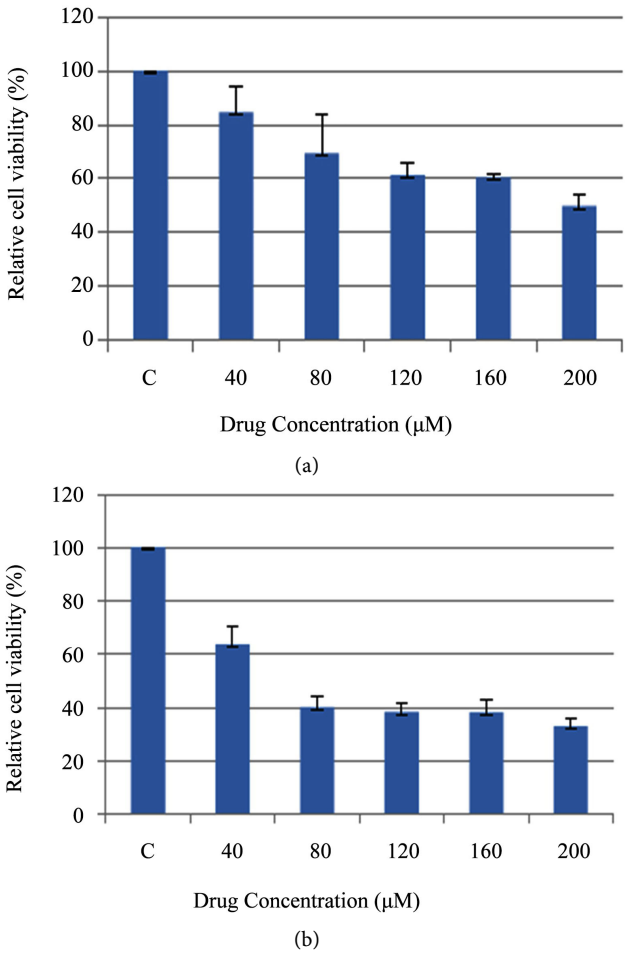
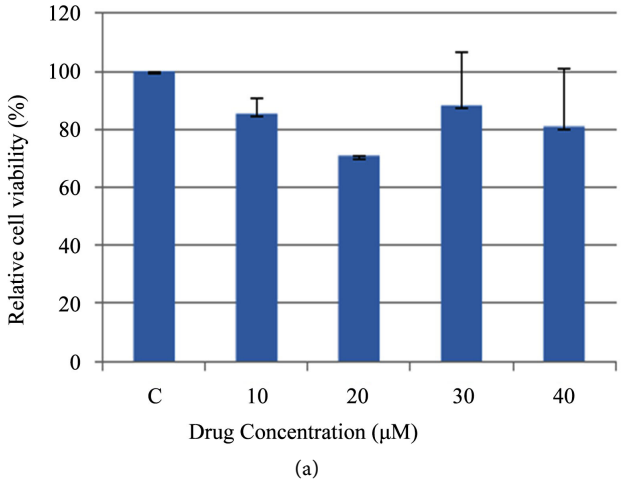
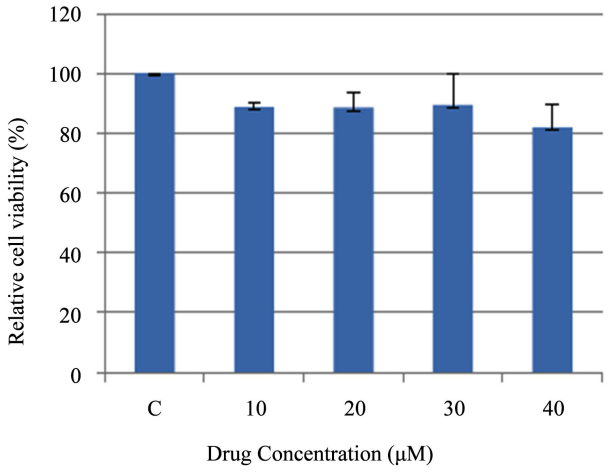


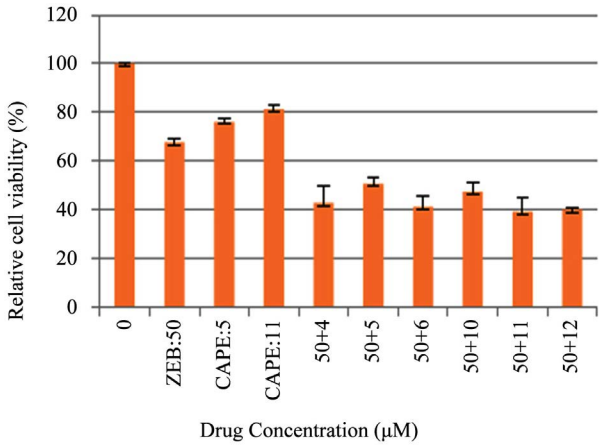
Figure 1. (a) Investigation of the effect of A. ZEB on cell viability in MCF-7 cell lines at 24 hours. (b) Investigation of the effect of ZEB on cell viability in MDA-MB-231 cell lines 24 hours.



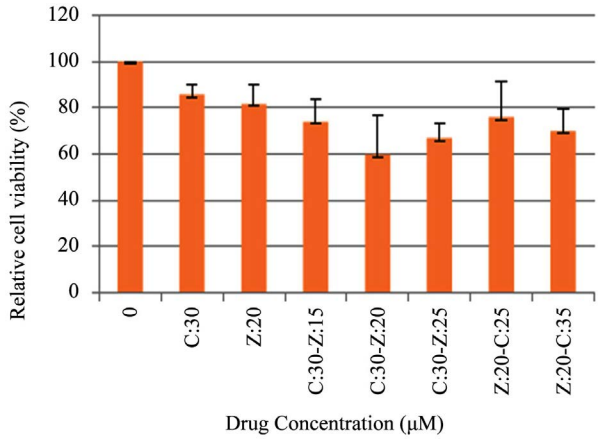


(b)

Figure 2. (a) Investigation of the effect of CAPE on cell viability in MCF-7 cell lines 24 hours. (b) Investigation of the effect of CAPE on cell viability in MDA-MB-231 cell lines 24 hours.



(a)



(b)

Figure 3. Investigation of the effect of combined doses of CAPE and ZEB on cell viability in MCF-7 cell lines for 24 hours. B. Investigation of the effect of combined doses of CAPE and ZEB on cell viability in MDA-MB-231 cell lines for 24 hours.

3.2. The Effect of CAPE, ZEB, CAPE + ZEB Combination on Cell Survival in MCF-7 and MDA-MB-231 Breast Cancer Cell Line

In order to demonstrate the effect of CAPE (20 - 40 μM) and ZEB on the proliferation of 200 μM MCF-7 and CAPE (120 μM) and ZEB (80 μM) MDA-MB-231 breast cancer cells, the cells were stained with trypan blue and then counted with hemocytometer. The counts showed that cell growth was decreased in time depending on time in both cell lines (Figure 4(a) and Figure 4(b)) ($p < 0.05$).

ZEB: 50 μM + CAPE: To demonstrate the effect of 5 μM administered drug combinations on the proliferation of MCF-7 breast cancer cells and the effects of ZEB 20 μM + CAPE 30 μM on the proliferation of MDA-MB-231 breast cancer cells, cells were stained with trypan blue and then counted with hemocytometer.

The counts showed that cell growth was decreased in time depending on time in both cell lines. When the data were examined, it was seen that combined therapy was effective in both cell lines without producing cytotoxic effect (Figure 5(a) and Figure 5(b)) ($p < 0.5$).

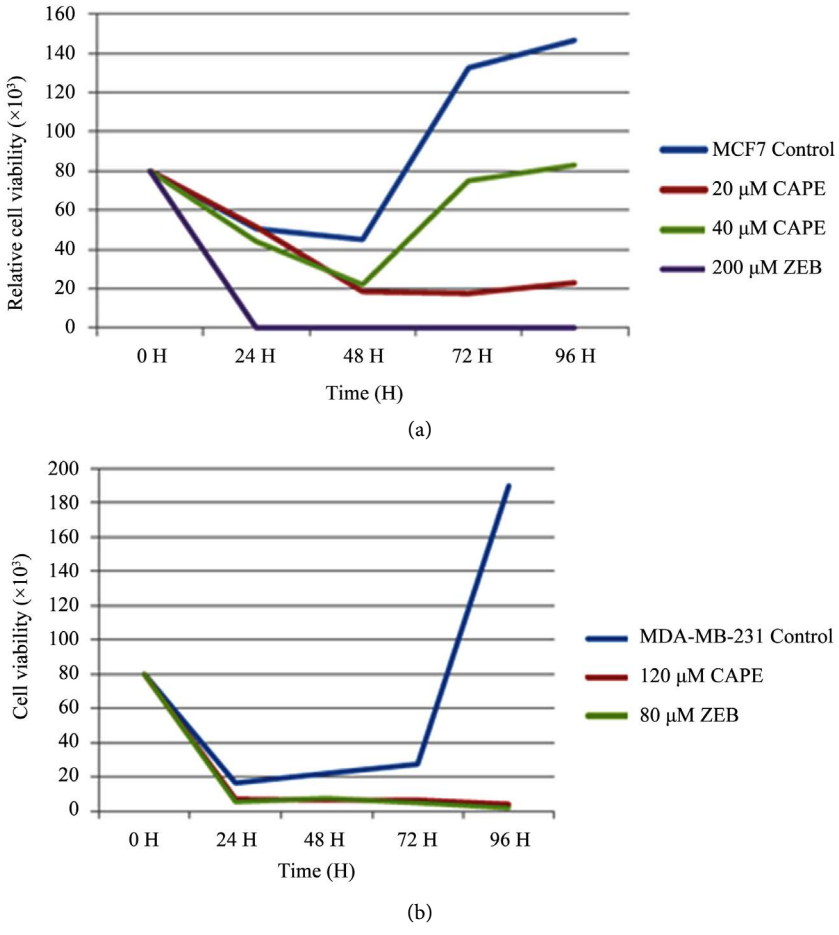


Figure 4. A demonstration of the effect of CAPE and ZEB on the proliferation of MCF-7 breast cancer cells. B. Effect of CAPE and ZEB on the proliferation of MDA-MB-231 breast cancer cells.

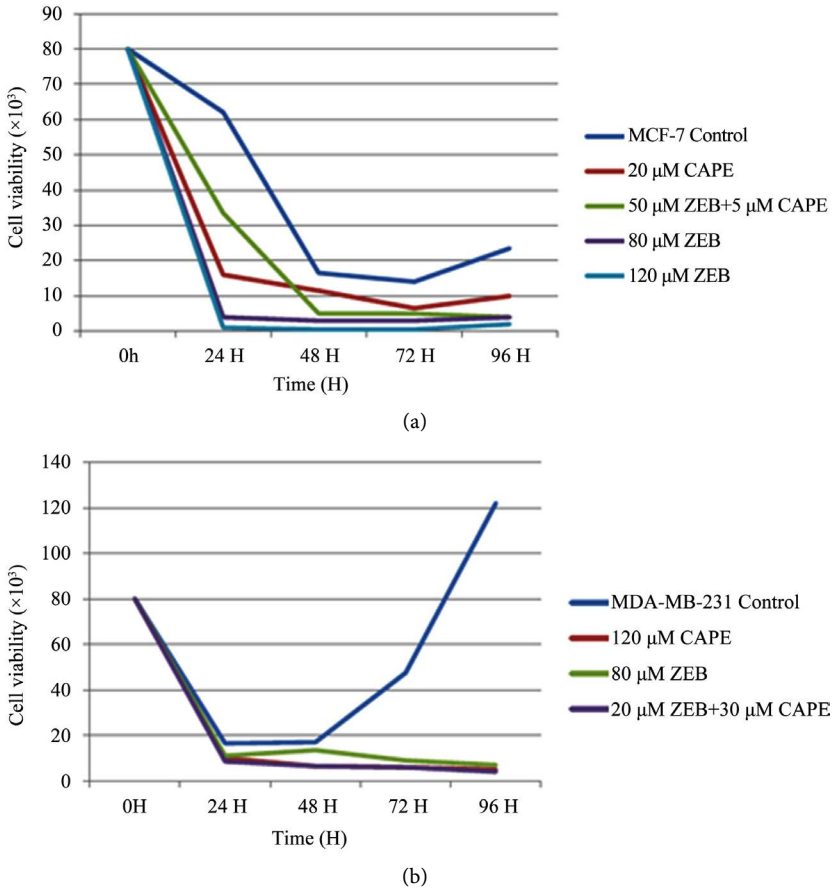


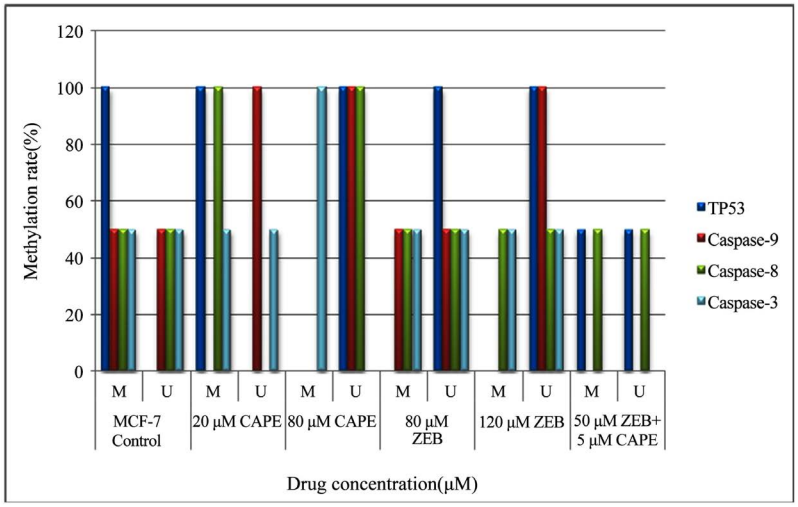
Figure 5. (a) Effect of combined drug administration of CAPE and ZEB on the proliferation of MCF-7 breast cancer cells (b). Effect of combined drug administration of CAPE and ZEB on the proliferation of MDA-MB-231 breast cancer cells.

3.3. Investigation of the Methylation Profile of Caspase-9, Caspase-8, Caspase-3 and TP53 Genes by MSP Method in MCF-7 and MDA-MB-231 Breast Cancer Cell Lines with CAPE, ZEB, CAPE + ZEB Combination

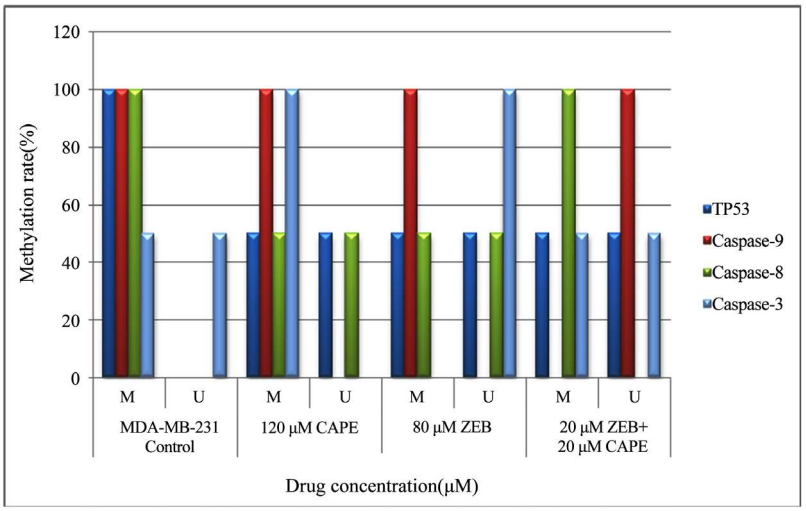
The methylation profile evaluation of the respective genes on 2 different cell lines used in the study is shown in **Figure 6** ($p < 0.5$).

As a result, it was concluded that the combined treatment in MCF-7 breast cancer cell lines was highly effective. However, it has been observed that the same doses of drugs in different genes may cause different methylation conditions. For example, the CAPE 80 μM dosage is closely related to the unmethylated condition in caspase-8 and caspase-9 in TP53 and caspase-3. On the other hand, in combination applications, the effect is constant and half is associated with methylation-unmethylation. This result is important in terms of the efficiency of the treatment.

In general, drugs administered on MDA-MB-231 cell lines did not have a methylation effect on caspase-8, but stimulation was carried out via the intrinsic pathway, which is the internal signaling pathway, or via a different signaling pathway. In addition, it is seen that the combined drug application is successful.



(a)



(b)

Figure 6. Methylation profile of TP53, Caspase-9, Caspase-8, Caspase 3genes in MCF-7 (a) and MDA-MB-231 (b) cell lines with combination of CAPE, ZEB, CAPE + ZEB.

4. Discussion

Although the monotherapy approach is still a very common treatment for many different types of cancer, this method is generally considered less effective than a combined therapy approach. Combined therapy can prevent toxic effects on normal cells and can also produce cytotoxic effects on cancer cells.

Zebularine is a DNMT inhibitor that exhibits anti-tumor effect against various types of cancer. CAPE significantly affects the viability of breast cancer cells and increases the effect of standard anti-cancer drugs, suggesting the potential role of bioactive compounds in chemotherapy. In this study, it was aimed to investigate the relationship between apoptosis induced by caspases and epigenetic regulation of the anticancer and antiproliferative effects of CAPE, ZEB and CAPE + ZEB combined applications in MCF-7 and MDA-MB-231 cell lines and the combined application of CAPE, ZEB single and CAPE + ZEB in MCF-7 and

MDA-MB-231 cell lines. The combined use of CAPE and ZEB drugs was applied for the first time in this study.

In this study, it was determined that single and combined application of ZEB and CAPE in two breast cancer cell lines reduced cell proliferation and induced apoptotic pathway (**Figures 2-4**). MDA-MB-231 breast cancer cell line MCF-7 cell line compared to MDA-MB-231 cell line because of the mutant TP53 gene is more susceptible to cytotoxic agents. In addition, one of the reasons for MCF-7 cells being less susceptible to the effect of ZEB is the low rate of growth in the cells due to the zebularine.

Billam *et al.* (2010) in his study showed that high concentrations of ZEB may cause cytotoxicity in cells, while inhibiting cell proliferation. Billam *et al.* found that ZEB, which is a DNMT inhibitor, is less toxic in many cell lines compared to other DNTMs. Therefore, it is thought that ZEB can be used alone or in combination with DNMT inhibitors such as other 5-aza-dC for prolonged administration [14]. In a study by Napso *et al.* (SCC-9 and SCC-25), 50 veM and 48 hours ZEB were applied in head and neck cancer cells. It has been shown that ZEB plays an important role in the induction of apoptosis due to time and reduces cell proliferation by approximately 20% [15]. Kaishan *et al.* (2016) found that ZEB inhibited proliferation of osteosarcoma cells and disrupted the interaction between DNMT1-G9a and increased apoptosis by suppressing methylation of the ARHI gene [16].

The combination of 50 μM ZEB + 5 μM CAPE combined dose of 50 μM in MCF-7 breast cancer cells after the 24-hour administration showed that the combined efficacy could be used effectively (**Figure 3(a)**). Rzepecka *et al.* (2017) found that IC_{50} values of CAPE MDA-MB-231 cells were found to be 27.84 50 M for 24-hour incubation and 15.84 MM for 48 hours [17]. CAPE has been confirmed to be cytotoxic for cells similar to that in this study. The nuclear factor of CAPE showed that it inhibited NFKB1. They also studied CAPE to confirm that death-inducing receptors were clustered. They demonstrated that CAPE induces apoptosis and that Fas death-inducing receptors are harvested by a Fas-L independent mechanism in MCF-7 cells [18].

(Omene *et al.* (2012)), *in vitro* and *in vivo* in the use of CAPE and taxol drug at appropriate dose concentrations were observed to inhibit cell growth when applied together [19]. Wadhwa *et al.* (2016) investigated the molecular mechanism of anticancer and anti-metastasis activities of CAPE. They examined the cytotoxicity of CAPE for a variety of human cancer cell lines, including A549, HT1080, G361, U2OS, MCF-7 and MDA-MB-231, and found that CAPE is cytotoxic for all cancer cells in the 5 - 100 μM range. Most of the IC_{50} value of 10 - 20 μM, while A549, a significant resistance (IC_{50} - 100 μM) and MCF-7 have determined that high sensitivity (IC_{50} - 5 μM) shows [20]. (Scott *et al.)* in his study AML-193 myeloid leukemia cells 250 - 500 μM with the application of ZEB 85% was shown to inhibit cell proliferation [21]. Omene *et al.* (2013) stated that estrogen receptor signaling plays a critical role in cell proliferation and

survival in breast cancer [22]. The triple negative breast cancer cell MDA-MB-231 is the ideal cell line due to its lack of estrogen receptor α and the small amount of estrogen receptor β. The ability of CAPE to bind estrogen receptors with its uncertain estrogenic effect has been demonstrated in previous studies. CAPE is a selective estrogen receptor modulator and has greater affinity for estrogen receptor α than estrogen receptor α. Therefore, estrogen receptor positive breast cancer cells are more sensitive to estrogen-related compounds. Given the similarity in structures between CAPE and estrogen, CAPE may be more effective than MDA-MB-231 (estrogen receptor-negative) cells in T47D (estrogen receptor positive) cells. According to this information, we can say that CAPE is more effective than MDA-MB-231 cells in MCF-7 cells that we use in our study.

The data obtained in combination with CAPE and DNMT inhibitor ZEB, which is known to be effective in apoptotic pathway in accordance with the literature, has been found to be effective in reducing the cytotoxicity caused by drugs. CAPE + ZEB combined drug therapy is thought to be promising for new treatments. This difference between the two cell lines makes MDA-MB-231 cells more susceptible to cytotoxicity of the drug because ER is negative. According to the data obtained in the literature, it was revealed that high dose concentrations of ZEB caused cytotoxic effects in the cell.

Recent studies have shown that the incidence of P53 mutations is different between the various types of cancer (~% 96 for serous ovarian cancer, <10% for thyroid cancer). The incidence of P53 mutation for primary breast carcinomas is 18% - 25% [23]. He stated that CAPE plays an important role in apoptosis and cell cycle. It has also been found in several studies that CAPE can reduce the expression of the MDR-1 gene and thus increase the susceptibility of cancer cells to chemotherapy, as the high expression and activity of the MDR1 gene causes the cancer cells to become resistant to treatment with many agents [24] [25].

The application of CAPE and ZEB to breast cancer cell lines induces p53 tumor suppressive activity and thus leads to apoptosis sensitivity of the cancer cell. In our study, 20 μM CAPE concentration was found to be associated with methylation of TP53 in MCF-7 cell lines (**Figure 6(a)**). The low dose ZEB concentration (80 μM) was found to increase the expression of the TP53 gene according to the high dose ZEB concentration (120 μM) (**Figure 6(a)**). These results obtained with 50 μM ZEB + 5 μM CAPE application were found to be related to the methylated and unmethylated status of the TP53 gene in half (**Figure 6(a)**). MDA-MB-231 breast cancer cell line with increasing the CAPE dose of the TP53 gene has been found to change from methylated to unmethylated state ($p < 0.05$) (**Figure 6(b)**). In a study conducted by You *et al.* (2014), it has been shown that TP53 activity, which is a tumor suppressor gene, has been increased in ZEB administered lung cancer cell line A549 and HPF cells [25]. In accordance with the literature, the data showed that the TP53 gene, which is inhibited by methylation in the MDA-MB-231 breast cancer cell line,

can induce apoptosis by re-expression by ZEB administration. In the new treatment approaches, the combination of drugs with different drugs due to their negative effects on toxicity is important for the health of the treatments.

Hurt *et al.* (2006) in a study similar to normal plasma cells found in hypermethylated myeloma cells P53 re-expression in the study of the application of 200 μM ZEB in compared to the control group increased 2-fold increase in P53 activity and also increased the amount of protein observed [26]. In a study by Signal *et al.* (1999), it is shown that the effect of 5-aza-Dc, a DNMTi drug in the MSP method-mediated P53 promoter, was associated with increased expression of P53 in malignant glioma cell lines, P53 mutant (T98G) and P53 non-mutant (U87MG) cell lines. In addition, mutations such as the conversion of cytosine (C) to uracil (U) by base deamination in cancer cells constitute 24% of the loss of expression in the TP53 gene, which has a major role in the formation of human solid tumors [27].

Eroglu *et al.* (2018) have shown that hypermethylation of tumor-related genes in breast cancer has an important role in carcinogenesis and tumor formation. They showed that GSTP1 and CDH1 tumor suppressor genes were hypermethylated in breast cancer tissues at 82% and 95%, respectively [28].

The effects of single and combined administration of ZEB and CAPE on caspase-9/-3 and -8, which are important factors related to apoptosis in cancer cells, were investigated. It was observed that the MCF-7 and MDA-MB-231 administered CAPE significantly increased the activation of caspase-9 gene ($p < 0.001$) (**Figure 6(a)** and **Figure 6(b)**). An epigenetically consistent result was obtained in stimulating the apoptotic pathway through caspase 9 by unmethylating the gene in the Zebularine, MCF-7 and MDA-MB-231 cancer cells. The 120 μM CAPE was applied to the MDA-MB-231 cells and the caspase-9 gene formed a methylated profile (**Figure 6(b)**). However, the combination of 20 μM ZEB + 30 μM CAPE on the caspase 9 gene was found to be more effective than the Zebularine, as opposed to the CAPE effect and a more efficient unmetilized condition, which was thought to be associated with a more active apoptotic stimulation and the efficacy of the combined application on the cells was higher (**Figure 6(b)**). In 2003, CAPE investigated the effect of P53 and P38 proteins on the application of C6 glioma cells and demonstrated that CAPE caused the release of cytochrome c from the mitochondria to the cytosol and activation of caspase-3 [29]. Jin *et al.*, in addition to CAPE administration on human myeloid leukemia U937, CAPE showed cytotoxic effect on cells and showed that this cytotoxic effect varies with both the applied CAPE concentration and the duration of the cells exposure [30].

When the data were analyzed, it was found that incubation of MCF-7 and MDA MB 231 cells with ZEB, CAPE and their combination triggered activation of caspase-9 and significantly increased caspase-3 activity (**Figure 6(a)** and **Figure 6(b)**). These results showed that combined therapy may be more effective in stimulating the apoptotic pathway in humans with breast cancer than in the ad-

ministration of drugs alone.

In MCF-7 cells, 80 μM CAPE concentration was found to be more effective than 20 μM CAPE concentration in activation of caspase-8 gene (**Figure 6(a)**). It can be said that the increase in dose concentration has a parallel effect with the activation of caspase-8 and is involved in the activation of the methylated gene by unmetrification. Combined administration of 50 μM ZEB + 5 μM CAPE has been found to be associated with halved methylated-unmethylated status of caspase-8 gene (**Figure 6(a)**). MDA-MB-231 breast cancer cells cannot be stimulated via caspase-8 ie extrinsic route. Because the MDA-MB-231 cells on a 120 μM CAPE and 80 μM ZEB concentrations alone when applied with a 50% methylated-unmetylated profile ($p < 0.01$), 20 μM ZEB + 30 μM CAPE combined application was found to be associated with the methylated situation (**Figure 6(b)**). It can be said that apoptotic stimulation is via the intrinsic (caspase-9) pathway.

The 80 μM CAPE application showed a 50% methylated-unmetylated profile of the caspase-8 gene in the cells. It was found that the caspase-3 gene was associated with methylene status in cells with 80 μM CAPE ($p < 0.01$) (**Figure 6(a)**). Although the caspase-9 gene associated with the intrinsic pathway of the drugs is related to the unmethylated state, the caspase-3 gene is associated with the methylated condition, and other mediators may be effective in stimulating the caspase-9 to induce apoptosis.

Tolba *et al.* (2013) found that CAPE combined with Paclitaxel and Docetaxel in prostate cancer was effective in reducing the IC50 volumes of these drugs, and showed that combined application with CAPE was 1.3 times more effective in the effectiveness of caspase-3 compared to drug administration alone [31]. Our study, which is supported by literature research, is to suggest that interactions of ZEB + CAPE combined drug therapy are more effective in cell proliferation and apopototic mechanism compared to ZEB and CAPE alone application to MDA-MB231 breast cancer cell line cells (**Figure 3(b)**). The explanation of the association of CAPE with apoptosis and the similarity of caspase gene induction processes in different cancer cell lines has been supported by literature studies.

In general, it has been observed that the drugs administered in MDA-MB-231 cell lines have no effect on the methylene status via caspase-8, according to the results obtained, it was thought that the stimulation was carried out via the intrinsic pathway, which is the internal signaling path, or through a different signaling pathway affected. It can also be said that the combined drug application is successful. Effective combination with low concentrations of dose ratios in the combined drug provides a more appropriate approach in terms of treatment.

Based on our findings, it was observed that CAPE, ZEB and CAPE + ZEB combined drugs inhibited MCF-7 and MDA-MB-231 cell proliferation and were more effective in apoptosis mechanism. It has been shown that the apoptosis-related genes were unmethylated and significantly increased apoptosis

by caspases. These findings suggest that ZEB's MCF-7 cells induce apoptosis along the intrinsic mitochondrial route, and that treatment with ZEB induces apoptosis in both MDA-MB-231 cells by intrinsic and extrinsic pathways. With the application of CAPE, caspase 3/7 in MCF-7 and MDA-MB-231 cells is shown to be effective for apoptosis activity and induce apoptosis events. Our findings are very important in combating the cytotoxic effect and combating cancer with combined therapy.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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