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RESEARCH ARTICLE

Caffeine May Improve the Chemotherapeutic Effect of Docetaxel by Inducing Unfolded Protein Response and Autophagy in Breast Cancer Cells

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SUMMARY

Breast cancer is the most frequently diagnosed cancer type among women. Chemotherapeutic agents are widely used in the treatment of breast cancer, but acquired drug resistance limits their effectiveness. Therefore, there is a continuing need for more effective treatment approaches with fewer side effects. Caffeine is one of the naturally occurring xanthines in coffee beans, caffeine is the most commonly used psychoactive substance worldwide. Numerous studies have highlighted the health benefits of coffee consumption, including reducing the risk of heart disease and certain cancers. Docetaxel is a second-generation antineoplastic agent of the taxane family and is widely used in the treatment of numerous cancers such as breast cancer. Herein, we evaluated the effect of caffeine and its combination with docetaxel on MCF-7 breast cancer cells. To test the effect of caffeine and its combination with docetaxel, we evaluated the autophagy, ubiquitin-proteasome system (UPS), unfolded protein response (UPR) signaling and apoptosis-related protein levels by immunoblotting. Cell viability was measured by WST-1 method. Morphological alterations in cells were evaluated in microscopical examinations. We found that caffeine remarkably induced UPR signaling, accelerated autophagic flux, and UPS-dependent protein turnover. Co-administration of caffeine and docetaxel strongly diminished the viability of MCF-7 cells by expanding the cytotoxic effect of docetaxel through accelerating the UPS-dependent protein turnover, induction of UPR and autophagy and apoptotic protein levels in a dose-dependent manner. Our results suggest that caffeine supplementation with docetaxel may expand the chemotherapeutic efficiency of docetaxel in breast cancer.

Key Words: *Autophagy, Apoptosis, Breast cancer, Caffeine, Unfolded Protein Response, Docetaxel*

Kafein, meme kanseri hücrelerinde katlanmamış protein tepkisini ve otofajiyi indükleyerek dosetakselin kemoterapötik etkisini artırabilir

ÖZ

Meme kanseri, kadınlar arasında en sık teşhis edilen kanser türüdür. Kemoterapötik ajanlar meme kanseri tedavisinde yaygın olarak kullanılmaktadır, ancak kazanılmış ilaç direnci etkinliklerini sınırlamaktadır. Bu nedenle, daha az yan etkiye sahip ve daha etkili tedavi yaklaşımlarına ihtiyaç vardır. Kahve çekirdeklerinde doğal olarak bulunan ksantinlerden biri olan kafein, dünya çapında en yaygın kullanılan psikoaktif maddedir. Çok sayıda çalışma, kalp hastalığı ve bazı kanser risklerinde azalma da dahil olmak üzere kahve tüketiminin sağlığa faydalarına dikkat çekmiştir. Dosetaksel, taksan ailesinden ikinci nesil bir antineoplastik ajandır ve meme kanseri gibi çok sayıda kanserin tedavisinde yaygın olarak kullanılmaktadır. Bu çalışmada kafein ve dosetaksel kombinasyonunun MCF-7 meme kanseri hücreleri üzerindeki etkisini değerlendirdik. Kafeinin dosetaksel ile kombinasyonunun etkisini test etmek için otofaji, ubikitin-proteazom sistemi (UPS), katlanmamış protein yanıtı sinyali (UPR) ve apoptozisle ilgili protein seviyelerini immünoblotlama ile inceledik. Hücre canlılığı WST-1 yöntemi ile ölçüldü. Hücrelerdeki morfolojik değişiklikler mikroskopi ile değerlendirildi. Kafeinin dikkat çekici bir şekilde UPR sinyalini uyardığını, otofajik akışı hızlandırdığını ve UPS'ye bağlı protein dönüşümünü uyardığını belirledik. Kafein ve dosetakselin birlikte uygulanması, dosetakselin sitotoksik etkisini UPS aracılı protein işlenmesini hızlandırarak, UPR ve otofajiyi uyararak ve apoptotik protein seviyelerini doz bağımlı bir şekilde arttırarak MCF-7 hücrelerinin canlılığını güçlü bir şekilde azalttı. Sonuçlarımız, dosetaksel ile kafein takviyesinin meme kanserinde dosetakselin kemoterapötik etkinliğini genişletebileceğini düşündürmektedir.

Anahtar Kelimeler: *Otofaji, Apoptozis, Meme Kanseri, Kafein, Katlanmamış Protein Yanıtı, Dosetaksel*

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INTRODUCTION

Breast cancer is described as a heterogeneous neoplasm accumulation around the mammary epithelium. It is divided into different subtypes according to the origin of cancer or receptor expression profiles and incidence, risk factors, prognosis and treatment options show differences between these subtypes. Breast cancer is the most frequently diagnosed cancer type among the women and second most common cause of death from cancer in the United States (Prat et al., 2015; Trayes & Cokenakes, 2021). Although there are different treatment options for breast cancer including radiation, endocrine therapy, immunotherapy, and chemotherapy, the need for novel therapeutic approaches continues (Trayes & Cokenakes, 2021). Acquired drug resistance is the main reason limiting the efficacy of chemotherapeutic agents (Trayes & Cokenakes, 2021). Thus, improving alternative therapeutic options such as natural products or combining these products with existing therapies may offer a good treatment option for breast cancer.

Xanthine is a derivative of purine alkaloids with a wide range of biologically active components (Jacobson et al., 1985). One of the naturally occurring xanthines in coffee beans, caffeine (1,3,7-trimethylxanthine) is the most widely used psychoactive substance around the world. Coffee, tea and beverages such as cola are the main vehicles for caffeine consumption in daily routine (Campa et al., 2005; Mitchell et al., 2015). Numerous studies have shown the health benefits of coffee intake including decrement in the risk of heart disease, endothelial dysfunction, inflammation, diabetes incidence and risk of some cancers (Andersen et al., 2006; Ding et al., 2014; Lopez-Garcia et al., 2006; A. Wang et al., 2016). Anti-cancer effects of caffeine have been studied for a long time. Studies have demonstrated a negative correlation between coffee consumption and the occurrence of various cancer types such as glioma and endometrial cancer (Pranata et al., 2022). Also, it has been reported that using a combination of caffeine and other chemotherapeutic agents such as paclitaxel, cisplatin, 5-fluorouracil and doxorubicin has enhanced antitumor effects in various cancer types such as breast, colorectal, lung and hepatocellular carcinoma (Mhaidat et al., 2014; Tomita & Tsuchiya, 1989; Z. Wang et al., 2019; Yamamoto & Tsuchiya, 2011). However, the effects of caffeine on various cancer types, including breast cancer, are still controversial and have not been achieved consensus. Thus, the possible synergistic effects of the chemotherapeutic agent docetaxel, which is frequently used in clinical applications for many cancer types, with caffeine were investigated in the present study.

Docetaxel also known as Taxotere, is a second-generation antineoplastic agent of the taxane family. It inhibits proliferation and causes cell death by disrupting microtubule functions and leading to cell cycle arrest at the G2/M phase (Jordan & Wilson, 2004). The efficacy of docetaxel has been shown in various types of cancer such as head and neck, gastric, non-small lung, hormone-refractory prostate and breast as well (Lyseng-Williamson & Fenton, 2005). Although it is commonly used in the treatment of breast cancer, adverse effects like unpredictable hypersensitivity reactions, neutropenia, fluid retention, and ocular toxicity limit its usage in advanced and recurrent cancer (Palmeri et al., 2008). Combined treatment of docetaxel with other agents has proven its effectiveness. Also, it is a valuable agent as it is a first-line treatment option for metastatic and aggressive cancer types (Hikita et al., 2021). Therefore, it is important to investigate the combined applications of docetaxel with natural products that do not have side effects that will increase its biochemical action. Herein, we aimed to investigate the possible anti-cancer effects of caffeine and its combined administration with docetaxel on MCF-7 breast cancer cells.

In the present study, we tested the effects of caffeine and its combination with docetaxel on unfolded protein response (UPR) signaling, the ubiquitin-proteasome system (UPS), autophagy and apoptotic cell death in MCF-7 human breast cancer cells. Our

results suggest that caffeine supplementation with docetaxel may expand the chemotherapeutic efficiency of docetaxel in breast cancer cells. However, the potential enhancer effect of caffeine on docetaxel needs to be broadly investigated with advanced experimental models in *in vitro* and *in vivo.*

MATERIAL AND METHODS

Materials

All cell culture reagents including fetal bovine serum (FBS), L-Glutamine and additional cell culture grade growth requirements were purchased from Biological Industries. Dulbecco's Modified Eagle Medium (DMEM) was purchased from LONZA Bioscience. Docetaxel (#9886), Bafilomycin A1 (#54645) and Staurosporine (#9953) were obtained from Cell Signaling Technology, Tunicamycin (sc-3506A) from Santacruz Biotechnology. Rabbit polyclonal antibodies PERK (#24390-1-AP), IRE1α (#27528-1-AP), XBP-1s (#24868-1-AP), ATF4 (#10835-1-AP), ubiquitin (#10201-1-AP), BiP/GRP78 (#11587-1-AP) and PARP1 (#13371-1-AP) were obtained from Proteintech. Rabbit monoclonal antibody against Hrd1 (#14773) and rabbit polyclonal cytochrome-c (#11940), eIF2α (#9722), phospho-eIF2α (Ser51) (#9721), caspase-3 (#9662), Beclin-1 (#3495), Atg5 (#12994), LC3-I/II (#12741), p62/SQSTM1 (#5114) were obtained from Cell Signaling Technology. Mouse monoclonal beta-actin antibody (#A5316) was purchased from Sigma Aldrich. HRP-conjugated goat anti-mouse (#31430) or goat anti-rabbit (#31460) IgG (H+L) was purchased from Pierce. High pure dimethyl sulfoxide (DMSO) was obtained from Genaxxon bioscience. Caffeine (C0750) was obtained from Sigma Aldrich.

Cell Culture

Human epithelial metastatic adenocarcinoma cell line, MCF-7 (HTB-22™) was obtained from American Type Tissue Culture (ATCC). Cells were routinely propagated in DMEM enriched with 10% FBS, 5 mg ml^{-1} penicillin/streptomycin, and 2 mM L-glutamine in a conventional cell culture condition, humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C. The absence of mycoplasma contamination was routinely confirmed by using MycoAlert™ Mycoplasma Detection Kit (Lonza).

Docetaxel was dissolved in DMSO (Serva #20385.01) to prepare the stock solution. Caffeine was prepared in cell culture media. The final concentration did not exceed 0.05% of the DMSO application amount. For this purpose, docetaxel was prepared at 2000X concentration. An equal amount of DMSO was applied as a vehicle application.

WST-1 Cell Proliferation Assay

The cell proliferation was measured by WST-1 based cell proliferation assay according to the manufacturer's instructions (Takara). The cells were seeded in 96-well plate (5000 cells/wells). Compound was applied to the cells for 24 or 48 h. To analyze the cell proliferation, 20 µl WST-1 reactive was added per well and cells were incubated for 2 hours under the conventional cell culture conditions. The absorbance was read at 450nm, with 600nm set as the reference wavelength by microplate spectrophotometer (BioTek, Epoch 2). The results were graphed to % fold change.

Microscopic Examination

Morphological changes of cells were performed using a phase-contrast inverted microscope (Sunny SopTop ICX41) and a digital camera system (OD400UHW). All photographs were taken with a 20x lens.

Protein Isolation and Immunoblotting

Cells were lysed in a Radioimmunoprecipitation assay (RIPA) buffer. After the removal of pellet by centrifugation at 14.000 rpm for 20 min at 4°C, total protein concentrations were determined from supernatant by bicinchoninic acid assay (BCA) kit (Takara). Generally, 30 µg of total protein was used for immunoblotting studies. Samples were denatured in 4x Laemmli buffer at 95°C for 5 min and protein samples were separated on 12% - 15% hand-cast polyacrylamide gels. Separated proteins were transferred

to an Immun-blot® polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in 5% nonfat dry milk in Phosphate-buffered saline (PBS) containing 0.1% Tween (PBS–Tween) for 1 h which was followed by incubation with the primary antibody in blocking buffer that was waited for 1-2 hours at room temperature. Protein bands were visualized by using clarity western enhanced chemiluminescence (ECL) substrate (Bio-Rad) and ChemiDoc XRS+ system (Bio-Rad).

Statistical Analysis

The results were presented as mean ± standard deviation (SD). The statistical significance of the differences between the groups was determined by the two-tailed equal variance student's t-test with a minimum confidence interval of 95% using GraphPad Prism 7 software. To determine differences among the groups, one-way ANOVA and Tukey's tests were used. The significant level was set at 5% ($p < 0.05$) for all tests.

RESULTS AND DISCUSSION

Caffeine remarkably induces UPR signaling and co-treatment with docetaxel exhibits a stronger effect on UPR in breast cancer cells.

Firstly, we determined the IC_{50} values of caffeine and docetaxel for 24 and 48 hours in MCF-7 cells (Figure 1a, b). We found that IC_{50} values of caffeine were 17.131mM ± 1.13 and 9.813mM ± 1.09 for 24 and 48 hours, respectively (Figure 1a). IC_{50} values for docetaxel were determined as 23.621nM ± 1,84 and 14.207nM ± 1.35 for 24 and 48 hours, respectively (Figure 1b). In further analysis, doses of 15nM for docetaxel and 1, 5 and 10mM for caffeine were used according to the cell viability assay data.

To test the effect of caffeine on UPR signaling, we treated the MCF-7 cells with 1, 5, 10mM caffeine for 24 hours and then tested the inositol-requiring enzyme-1α (IRE1α) and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) branches of UPR proteins including total-eukaryotic translation initiation factor 2 (eIF2α), phospho (Ser51)-eIF2α, activating transcription factor 4 (ATF4) and PERK; IRE1α and spliced form of X-box binding protein 1 (XBP-1s) proteins and also ER stress related chaperone binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78) by immunoblotting. Our data indicated that caffeine treatment gradually increased protein expression level of all tested UPR members in a dose-dependent manner (Figure 1c). Similar to caffeine, docetaxel was determined to stimulate UPR proteins compared to the control group (Figure 1d). Moreover, co-administration of caffeine with docetaxel more strongly induced the expression of all tested proteins in a dose-dependent manner compared to docetaxel alone administration (Figure 1d). These results suggested that caffeine positively regulated UPR signaling and its combined treatment with docetaxel expanded the chemotherapeutic efficiency by synergistically affecting UPR signaling.

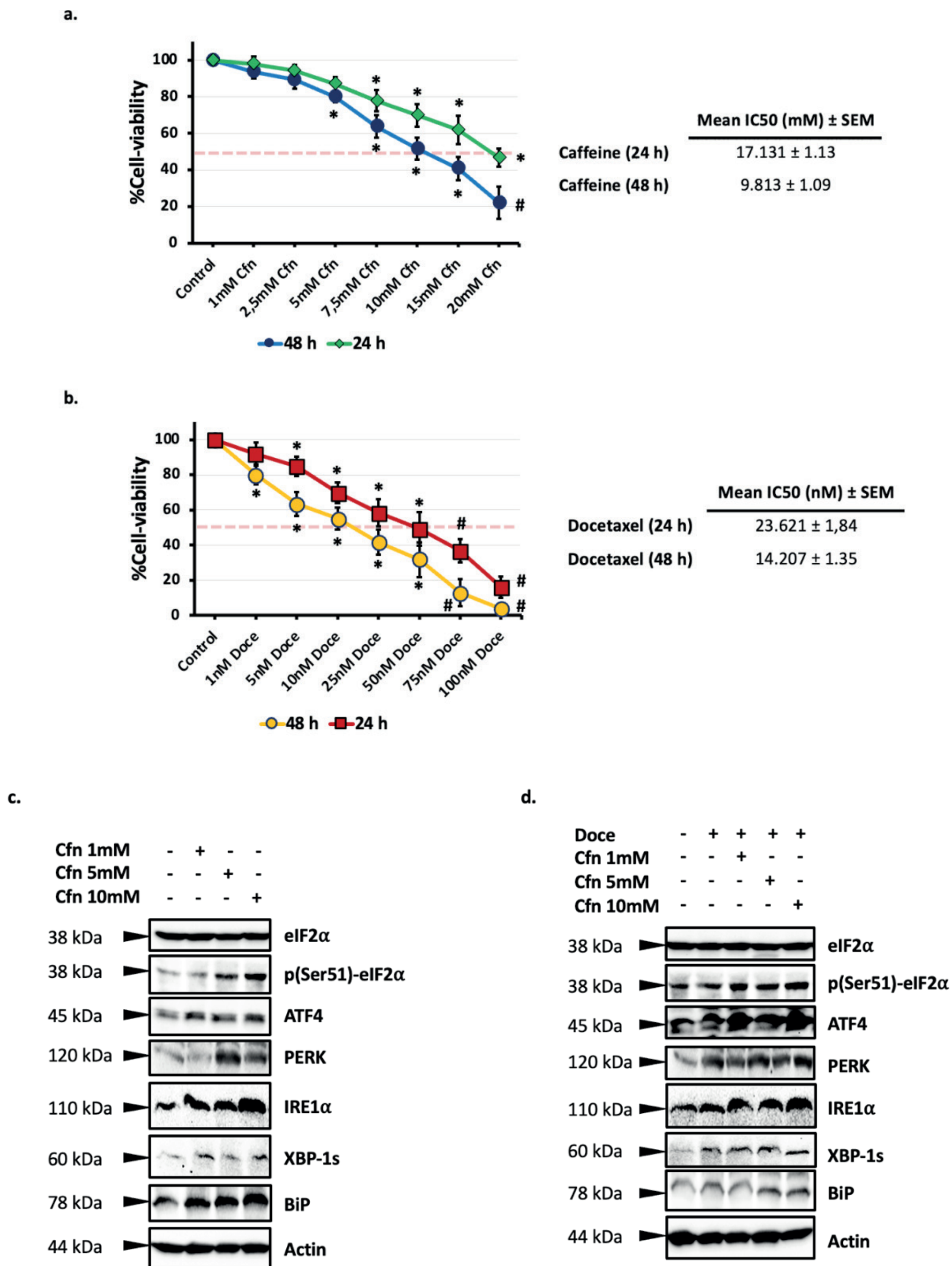

Figure 1. Evaluation of caffeine and its combination with docetaxel on UPR related proteins. (a, b) The effect of caffeine and docetaxel on the cell viability of MCF-7 cells was analyzed using the WST-1 assay. IC_{50} values were calculated using GraphPad Prism 7 Data represented as mean ± SE of three independent experiments made in three replicates. (c) MCF-7 cells were treated with a vehicle or 1, 5 and 10mM for 24 hours and then UPR related protein levels were analyzed by immunoblotting using antibodies raised against them. (d) Combine treatment of caffeine as indicated doses and 15nM docetaxel for 24 hours and then the expression level of UPR proteins was analyzed by immunoblotting. Beta-actin was used as a loading control.

Caffeine dose-dependently decreased the poly-ubiquitination and it exhibits a synergistic effect with docetaxel in breast cancer cells

To evaluate the effect of caffeine on protein turnover, we tested the poly-ubiquitination pattern in MCF-7 cells by immunoblotting. We found that caffeine treatment dose-dependently reduced the poly-ubiquitination levels compared to control group (Figure 2a). Next, we examined the levels of HMG-CoA reductase degradation 1 (Hrd1)/Synoviolin 1 (SYVN1), which is the major E3 ubiquitin ligase enzyme that plays a role in ER-associated degradation (ERAD), we determined that Hrd1 protein levels increased in a dose-dependent manner (Erzurumlu & Ballar, 2017)(Figure 2a). Combined treatment of caffeine with docetaxel more strongly induced Hrd1 protein levels compared to the docetaxel alone group (Figure 2b). Also, the poly-ubiquitination pattern was more robustly decreased by the caffeine-docetaxel group in a dose-dependent manner (Figure 2b).

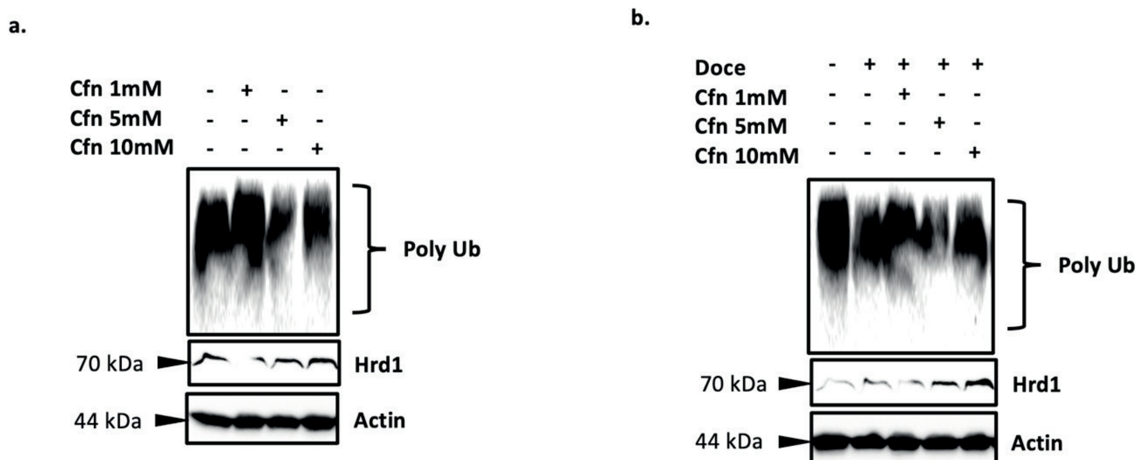

Figure 2. The effect of caffeine and co-treatment with docetaxel on ERAD E3 ligase Hrd1 and poly-ubiquitination pattern. (a) MCF-7 cells were treated with a vehicle or as indicated doses of caffeine for 24 hours and Hrd1 and poly-ubiquitination levels were analyzed by immunoblotting. (b) As indicated doses of caffeine and its combination with docetaxel were applied to the MCF-7 cells for 24 hours and target proteins were analyzed by immunoblotting. Beta-actin was used as a loading control.

Caffeine ameliorates the biochemical effect of docetaxel by autophagic induction

We investigated the effects of caffeine on basal autophagic activity in MCF-7 by testing the Beclin-1, autophagy related 5 (Atg5) protein levels, p62/sequestosome 1 (SQSTM1) turnover and microtubule associated protein 1 light chain 3 (LC3)-I conversion to LC3-II by immunoblotting. Our data indicated that caffeine administration gradually increased Beclin1 levels whereas we did not determine any alteration level of Atg5 at administered all doses of caffeine (Figure 3a). p62/SQSTM1 levels slightly decreased by caffeine treatment in a dose-dependent manner. More strikingly, LC3-I conversion to LC3-II remarkably accelerated with caffeine (Figure 3a).

Next, we evaluated the combined effect of caffeine with docetaxel on autophagy in MCF-7 cells. Docetaxel administration does not affect the Atg5, p62/SQSTM1, LC3-I and LC3-II protein levels whereas Beclin1 expression was decreased (Figure 3b). Co-treatment caffeine with docetaxel remarkably increased Beclin1 and Atg5 protein levels in a dose-dependent manner. Moreover, the turnover of p62/SQSTM1 was accelerated with combined treatment. Additionally, LC3-I to LC3-II conversion was dramatically induced by co-administration compared to the docetaxel-treated group (Figure 3b). In this assay system, BafA1, which is a well-known autophagy inhibitor used as a positive control (Yoshii & Mizushima, 2017). As expected, the steady-state level of LC3-I and LC3-II increased due to the inhibition of the fusion of the autophagosome with the lysosome by 1μM BafA1 treatment (Figure 3b).

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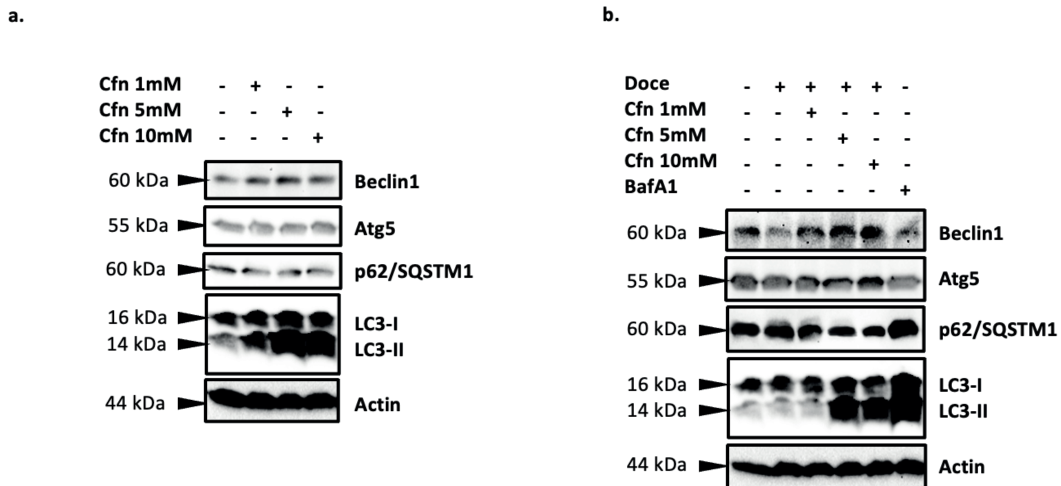
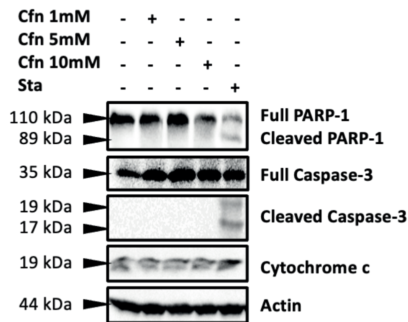
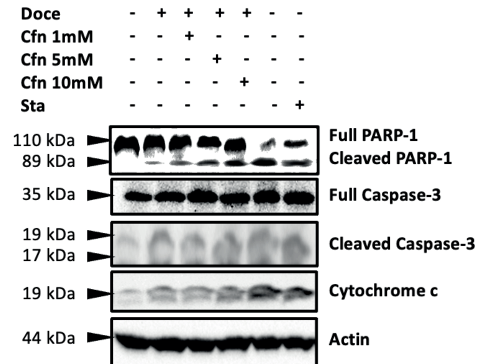


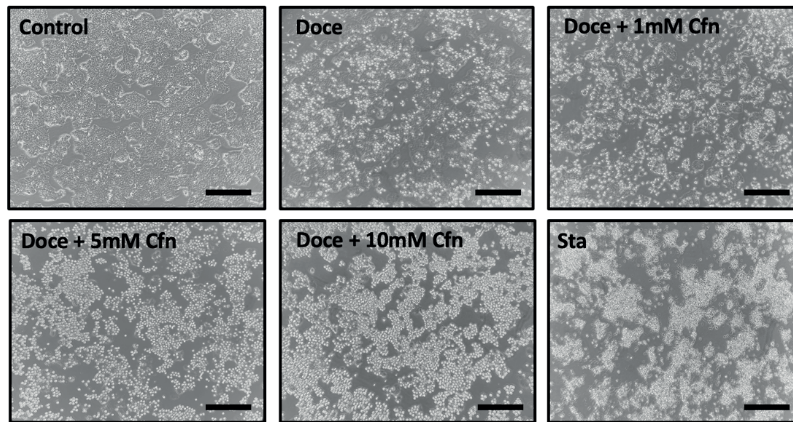
Figure 3. The effect of caffeine and docetaxel on autophagy. (a) Autophagy protein levels including Beclin-1, Atg5, p62/SQSTM1, LC3-I and LC3-II were analyzed by immunoblotting. (b) Autophagy-related protein levels were examined in MCF-7 cells treated with caffeine or co-treatment with docetaxel. Beta-actin was used as a loading control. 1µM BafA1 was used as an autophagic inhibitor control.

Caffeine synergistically enhanced the apoptotic effect of docetaxel

To investigate the cell-death-related effect of caffeine on MCF-7 cells, we tested the Poly(ADP-Ribose) Polymerase 1 (PARP-1) and caspase-3 cleavage, and cytochrome-c levels by immunoblotting. In this assay system, staurosporine, which is a well-characterized apoptotic inducer used as a positive control of apoptotic cell death (Malsy et al., 2019). Our data indicated that caffeine treatment at indicated doses has no apoptotic effect on MCF-7 cells (Figure 4a). Next, we tried its combination with docetaxel, we found that co-treatment of caffeine with docetaxel strongly induced PARP-1 cleavage in a dose-dependent manner compared to docetaxel. We obtained similar results in the caspase-3 cleavage product. (Figure 4b). Moreover, cytochrome-c levels were remarkably up-regulated by combined treatment compared to docetaxel alone administration (Figure 4b). Microscopic examination results showed that combined treatment of docetaxel with caffeine more strongly induced shrinking and the levels of dead cell debris compared to docetaxel group (Figure 4c). Additionally, cell proliferation assay data indicated that co-treatment of docetaxel and caffeine significantly decreased % cell viability (Figure 4d) (Table 1). Collectively these results indicated that caffeine expanded the anti-tumorigenic effect of docetaxel by more efficiently inducing apoptotic cell death. Additionally, we calculated the combination index (CI) value for determining whether the effect of the combined treatment of docetaxel and caffeine synergistically manner. Also, caffeine exhibited a synergistic effect at 5 and 10mM doses with docetaxel on MCF-7 cells (Table 2).







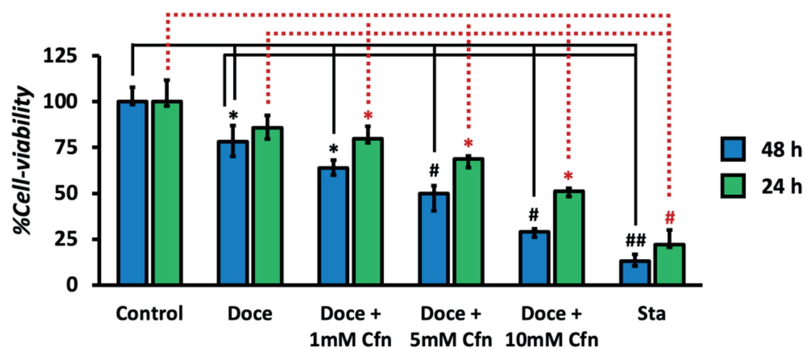


Figure 4. The effects of co-treatment of caffeine with docetaxel on cell-death related proteins. (a) Apoptotic protein levels were analyzed as described Figure 1. Staurosporine (1nM) was used as a positive control of apoptotic cell death. (b) Cells were treated with a vehicle or combined treated with docetaxel and caffeine as indicated doses for 24 hours and target proteins were analyzed by immunoblotting. Beta-actin was used as a loading control. (c) MCF-7 cells were treated with docetaxel and its combination with caffeine and the morphological alterations were visualized in inverted microscope and photographed. Staurosporine was used as a positive control. The scale bar represents 25µm. (d) Cells were treated with vehicle and indicated doses of caffeine or its combination with docetaxel for 24 and 48 hours. % cell viability was analyzed by WST-1 assay. Data represented as mean ± SE of three independent experiments made in three replicates. *$p < 0.05$, #$p < 0.001$, ##$p<0.0001$.

Table 1. The % cell viability values of Docetaxel and its combination with Caffeine.

	% Cell Viability (24h)	% Cell Viability (48h)
Control	100 ± 7.11	100 ± 4.67
Doce	85,723 ± 6.41	78,105 ± 8.37
Doce + 1mM Cfn	79,831 ± 4.46	63,832 ± 4.016
Doce + 5mM Cfn	68,732 ± 3.24	49,921 ± 6.87
Doce + 10mM Cfn	51,084 ± 2.24	28,943 ± 2.24
Sta	22,161 ± 4.71	13,051 ± 3.17

Data represent mean % cell viability value with ± standard deviations.

Table 2. Values of combination index (CI) for docetaxel-caffeine interactions.

	CI value ± SEM	Combine Effect
Docetaxel [15nM] + Caffeine 1mM	1.03 ± 0.03	Additive
Docetaxel [15nM] + Caffeine 5mM	0.83 ± 0.11	Synergistic
Docetaxel [15nM] + Caffeine 10mM	0.65 ± 0.13	Synergistic

CI greater than 1.1 denotes an antagonist effect; CI between 0.99-1.1 indicates an additive effect; CI value less than 0.99 indicate a synergistic effect. Data represent mean CI value with ± standard deviations. Calculations of CI were calculated with GraphPad Prism 7 software.

Caffeine is the most common consumed dietary ingredient around the world. It has various potent biochemical activities such as antioxidant, anti-inflammatory and anti-cancer. Studies demonstrated that it is effective against various diseases such as Alzheimer's disease, Parkinson's disease and cancer in *in vitro* and *in vivo* (Campa et al., 2005; Mitchell et al., 2015; Pranata et al., 2022). Also, it has been shown to give effective results when used as an auxiliary ingredient with traditional drugs and chemotherapeutics (Mhaidat et al., 2014; Tomita & Tsuchiya, 1989; Z. Wang et al., 2019; Yamamoto & Tsuchiya, 2011). In the present study, we evaluated the effect of caffeine and its combination with docetaxel on MCF-7 breast cancer cells. Docetaxel which belongs to the taxane family is one of the commonly used antineoplastic agents in the treatment of breast cancer. It is also effective in the treatment of other cancer types including gastric cancer, head and neck cancer and non-small cell lung cancer. However, long-term usage of docetaxel in treatments causes systemic disturbances, therefore, its therapeutic efficiency is limited clinically (Lyseng-Williamson & Fenton, 2005).

The goal of our study is to test the effects of caffeine on UPS, autophagy and UPR signaling, which are critical mechanisms for breast and other cancer types and they often have increased activity in breast cancer cells. Also, the possible synergistic effects of the use of caffeine together with docetaxel are evaluated through these mechanisms.

Today, it is known that UPS, UPR, and autophagy work together to regulate physiologically cellular homeostasis. UPS is the main proteolytic system that is crucial in the regulation of many cellular processes by promoting protein degradation and homeostasis (Erzurumlu & Ballar, 2017; Mata-Cantero et al., 2015). The endoplasmic reticulum-associated degradation (ERAD) mechanism, which is one of the most effective protein degradation systems in the ER, ubiquitinates the substrate molecules to target 26S proteasome by ubiquitin ligase enzymes (Erzurumlu & Ballar, 2017). Impairment of UPS has been associated with various diseases such as diabetes, Alzheimer's disease, Parkinson's disease, hemophilia, and numerous cancer types (Erzurumlu & Ballar, 2017). The variable factors such as deficiency of nutrients, toxic stimulation and im-

balance of metabolic Ca^{+2} can trigger stressful conditions in ER and these conditions induce called "ER stress" signaling (Madden et al., 2019). Induction of ER stress leads to activation of the UPR mechanism, supporting the restoration of ER homeostasis. UPR is coordinated through the activation of ER-membrane localized three transmembrane proteins, PERK, IRE1α, and activating transcription factor 6 (ATF6) sensor protein and their down-regulators (Madden et al., 2019). To evaluate the effects of caffeine on UPR signaling, we tested effector proteins of UPR, including p(Ser51)-eIF2α, total-eIF2α, ATF4, and PERK and also IRE1α branch, XBP-1s and IRE1α protein levels. Caffeine treatment increased expression of all tested UPR proteins in a dose-dependent manner. Also, to understand the relationship between UPR induction and ER stress, we examined the levels of BiP/GRP78, a well-known stress protein. Our results showed that caffeine administration increased the protein level of BiP/GRP78 in a dose-dependent manner (Figure 1c). These results suggest that caffeine induces ER stress in breast cancer cells and activation of the UPR occurs in this way. In addition, the dose-dependent decrease in the poly-ubiquitination pattern in the cells with caffeine administration and the dose-dependent increase in the levels of Hrd1/SYVN1, one of the major ubiquitin ligase enzymes associated with ERAD, suggests that caffeine decelerates the UPS in breast cancer cells (Figure 2a). Similar results were obtained with the co-administration of caffeine and docetaxel. Co-treatment more strongly decreased the poly-ubiquitination state, whereas increased Hrd1 levels and induced the UPR signaling in MCF-7 cells (Figure 1b, 2b).

Autophagy is a conserved program that is comprised of initiation and membrane nucleation, phagophore formation and expansion, fusion with the lysosomes and degradation (Glick et al., 2010). It controls the level of damaged organelles, long-lived and misfolded proteins (Glick et al., 2010). To investigate effects of caffeine on autophagy, we tested critical regulator proteins Beclin-1 and Atg5, turnover of p62/SQSTM1, which is well known autophagic cargo protein and lipidation of LC3 considered necessary for autophagosome formation.

Our results indicated that caffeine increased Beclin1 levels and did not cause changes in Atg5 levels. Also, it slightly decreased p62/SQSTM1 levels, whereas it remarkably induced LC3-I to LC3-II conversion in a manner dose-dependently (Figure 3a). These results observed that caffeine accelerated the autophagic flux in breast cancer cells. Moreover, co-treatment of caffeine and docetaxel led to an increment level of Beclin1 and Atg5 compared to control or docetaxel alone. More strikingly, the combined treatment of caffeine and docetaxel remarkably accelerated the p62/SQSTM1 turnover and also strongly increased LC3-I to LC3-II conversion. In this assay system, BafA1 was used as an autophagic blocker, which blocks the fusion of autophagosomes with lysosomes, and as expected, it increased p62/SQSTM1 accumulation and LC3-II levels (Figure 3b). Collectively these results suggest that caffeine alone positively regulates autophagic activity, and when combined with docetaxel, it synergistically stimulates autophagic flux in breast cancer cells. Autophagy is also known as the type 2 cell death program, highly increased autophagic activity leads to cell death (Kroemer & Levine, 2008). Consistent with the cell death findings observed in our microscopic results, increased autophagic activity may have led to the death of MCF-7 cells (Figure 4c).

Next, we tested the effect of caffeine and its combination with docetaxel on apoptotic cell death. We observed caffeine administration did not induce any apoptotic cell death-associated protein levels, including PARP-1 and caspase-3 cleavage, cytochrome-c (Figure 4a).

The evaluation of the cleavage forms of caspase-3 and PARP-1 is widely used for understanding apoptotic cell death in *in vitro* and *in vivo* studies (Cohen, 1997; Lazebnik et al., 1994). Also, cytochrome c is a component of the mitochondrial electron transport chain and is an essential parameter for the evaluation of extrin-

sic apoptotic cell death (Liu et al., 1996). Our findings indicated that co-treatment of caffeine with docetaxel strongly induced 89 kDa cleavage fragment of PARP-1, which is one of the main targets of caspase-3 and cleavage form of caspase-3, which is a critical executioner of apoptosis in a dose-dependent manner (Cohen, 1997; Lazebnik et al., 1994). Consistent with these data, co-treatment synergistically increased cytochrome-c levels in MCF-7 cells (Figure 4b).

It is frequently stated as a fact that chemotherapeutic agents trigger cell death through apoptotic mechanisms. However, recent studies indicated that these agents could activate complex cell death modes by affecting different cellular signaling mechanisms, including necrosis, ferroptosis and autophagic cell death (Gao et al., 2022). Herein we tested the possible effects of the natural product caffeine in breast cancer cells and also determined the synergistic effect of its combination with docetaxel. Our results suggest that the concomitant use of caffeine and docetaxel affects breast cancer cells by autophagic induction and intrinsic/extrinsic apoptotic pathway. The mode of action of the combination of caffeine with docetaxel was illustrated in Figure 5.

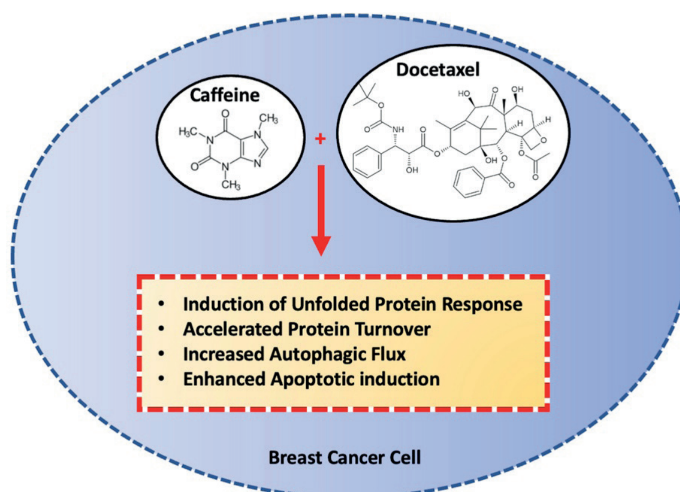


Figure 5: Schematic illustration of the mode of action of caffeine with docetaxel in breast cancer cells.

CONCLUSION

Testing the well-known traditional chemotherapeutics and their combination with natural products is a very valuable effort for increasing their utilization and establishing less toxic treatment protocols. The present study suggests that caffeine, which is frequently consumed in the community as part of the daily diet, may increase the chemotherapeutic efficacy of docetaxel in breast cancer treatment.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Y.E. initiated and directed the project, designed, and conducted the experiments, analyzed, and interpreted the results, and wrote the manuscript. D.C., H.K.D. and E.A. assisted experimental studies. All

correspondence and requests for materials should be addressed to Y.E. All authors have read and approved the final version of the article.

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