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The effect of DEBIO 1143 usage alone or in combination with tamoxifen on estrogen receptor positive breast cancer cell lines

DEBIO 1143'ün tek başına veya tamoxifenle kombinasyonunun östrojen reseptörü pozitif meme kanseri hücre hatlarındaki etkisi

Berrin Tuğrul, Merve İşseven

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Abstract

Purpose: To investigate the effect of increased concentrations of tamoxifen (TAM) and DEBIO 1143 (AT-406) administered alone or in combination on cells in MCF-7 and BT-474 estrogen receptor positive (ER+) breast cancer cell lines.

Materials and methods: The effect of tamoxifen and DEBIO 1143 administered alone or in combination on cell viability in MCF-7 and BT-474 cell lines at the 72nd hour was assessed by the XTT test. Multi-parameter apoptosis assay kit was used to display the effect of the alone/combination of TAM and DEBIO 1143 on both cell lines. Fluorescence microscobic analysis was performed.

Results: The IC50 value of TAM was 3.8±0.6 micromolar (μM) and 18.9±6.7 μM in the MCF-7 and BT-474 cell lines, respectively. The IC50 value of DEBIO 1143 was 15±0.5 μM in the MCF-7 cell line. The results related to drug combination were statistically significant for both cell lines (p<0.001). The decrease in cell viability was not associated with apoptosis.

Conclusion: In ER+breast cancer cell lines, the combined doses of the TAM and DEBIO 1143 reduced cell viability more than their administration alone. Combined administrations in both cell lines were concluded in a synergistic effect. Further research is needed to determine which cell death type other than apoptosis is associated with a reduction in cell viability caused by combined administration.

Key words: DEBIO 1143, ER+breast cancer, SMAC mimetic, tamoxifen.

Tuğrul B, İşseven M. The effect of DEBIO 1143 usage alone or in combination with tamoxifen on estrogen receptor positive breast cancer cell lines. Pam Med J 2020;13:9-18.

Özet

Amaç: Tamoxifen (TAM) ve DEBIO 1143 (AT-406)'ün artan konsantrasyonlarının tek başına veya birlikte uygulanmasının, MCF-7 ve BT-474 östrojen reseptörü pozitif (ER+) meme kanseri hücre hatlarındaki etkisini araştırmak.

Gereç ve yöntem: XTT testi, tamoxifen ve DEBIO 1143'ün tek başına veya birlikte kullanımın 72 saatlik MCF-7 ve BT-474 hücre hatlarındaki hücre canlılığı üzerine olan etkisini belirlemek için kullanıldı. TAM ve DEBIO 1143'ün tek başına / kombinasyonunun her iki hücre hattı üzerindeki etkisini göstermek için çok parametreli apoptoz analiz kiti kullanıldı ve floresan mikroskobik görüntüleme yapıldı.

Bulgular: TAM'ın IC50 değeri MCF-7 ve BT-474 hücre hatlarında sırasıyla 3,8±0,6 mikromolar (μM) ve 18,9±6,7 μM idi. DEBIO 1143'ün IC50 değeri, MCF-7 hücre hattında 15±0,5 uM idi. İlaç kombinasyonuna bağlı sonuçlar her iki hücre hattı için istatistiksel olarak anlamlıydı (p<0,001). Hücre canlılığının azalması apoptozis ile ilişkili bulunmadı.

Sonuç: ER+meme kanseri hücre hatlarında, kombinasyon halinde uygulanan TAM ve DEBIO 1143 dozlarının, tek başına uygulanmalarına göre hücre canlılığını daha fazla azalttığı belirlenmiştir. Her iki hücre hattında kombinasyon dozlarının uygulanması sinerjik bir etki göstermiştir. Kombine uygulamanın sebep olduğu hücre canlılığındaki azalmanın, apoptoz dışındaki hangi hücre ölüm tipi ile olduğunu belirlemek için ileri araştırmalara ihtiyaç vardır.

Anahtar Kelimeler: DEBIO 1143, ER+meme kanseri, SMAC mimetik, tamoxifen.

Tuğrul B, İşseven M. DEBIO 1143'ün tek başına veya tamoxifenle kombinasyonunun östrojen reseptörü pozitif meme kanseri hücre hatlarındaki etkisi. Pam Tıp Derg 2020;13:9-18.

Berrin Tuğrul, Assit. Prof. Dr. Molecular Biology Division, Department of Biology, Faculty of Science and Letters, Manisa Celal Bayar University, 45140, MANİSA, e-mail: berrin.tugrul@yahoo.com (orcid.org/0000-0003-0844-7766) (Sorumlu yazar)

Merve İşseven, MSc, Molecular Biology Division, Department of Biology, Faculty of Science and Letters, Manisa Celal Bayar University, 45140, MANİSA, e-mail: issevenmerve@hotmail.com (orcid.org/0000-0001-8302-7943)

Introduction

Breast cancer is one of the heterogeneous disease groups and leading causes of death among women worldwide [1]. More than 60% of human breast cancers are estrogen receptor (ER) positive. Tamoxifen (TAM), a selective estrogen receptor modulator (SERM), is the most common endocrine therapy used in this type of cancer. Approximately 50-60% of patients with estrogen receptor positive (ER+) breast cancer benefit from TAM, while others do not respond. In cells overexpressing human epidermal growth factor receptor 2 (HER2), TAM resistance may develop [2].

There has been a growing interest in combination therapies performed with chemotherapeutics and second mitochondrial-derived activator of caspase (SMAC) mimetics in different cancer types. SMAC mimetics are inhibitors of endogenous inhibitor of apoptosis protein (IAP) acting as an IAP antagonist. Several studies have demonstrated that both monovalent and bivalent SMAC mimetics increased the antitumor activity of other anticancer agents, promoted apoptosis in human cancer cell lines *in vitro* and displayed tumor healing capacity in animal models [3].

DEBIO 1143 (AT-406) is an orally available SMAC mimetic that effectively targets X-linked inhibitor of apoptosis protein (XIAP) and cellular apoptosis protein 1/2 (cIAP1/2) [4]. *In vitro* studies on various human cancer cell lines revealed that it inhibits cancer cell proliferation effectively. In *in vivo* xenograft tumor studies conducted with mice, rats, dogs and non-human primates, DEBIO 1143 has been shown to induce apoptosis. DEBIO 1143 is currently being investigated in phase 1 clinical trials in the treatment of human cancers [5]. Our literature research demonstrated no publication related to the combined therapy of TAM and DEBIO 1143 in estrogen receptor positive breast cancer cell lines.

In the present study, it was aimed to investigate the effects of co-administration of TAM and DEBIO 1143 on MCF-7 (Luminal A type) and BT-474 (Luminal B type) estrogen receptor positive breast cancer cell lines and to determine the cell death type associated with it.

Materials and methods

Materials

MCF-7 and BT-474 human breast cancer cell lines were obtained from Experimental Research and Application Center Celal Bayar University, and Department of Genetics and Bioengineering, Yeditepe University, respectively.

Cell culture

MCF-7 cell line was cultured in the Dulbecco's Modified Eagle's medium (DMEM) containing L-glutamine by adding 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin [6]. The BT-474 cell line was cultured in the DMEM containing L-glutamine by adding 10% FBS, 1% Penicillin/Streptomycin and 10 microgram per milliliter (µg/mL) insulin [7]. Cell lines were proliferated in a humidified incubator at 37°C with 5% CO_2.

Preparation of stock solutions of drugs

A 1 millimolar (mM) stock solutions of TAM powder (Sigma, Germany) and DEBIO 1143 (ApexBio, Taiwan) were prepared by dissolving them in Dimethyl sulfoxide (DMSO). After the stock solutions were passed through a 0.22 micrometer (µm) filter, they were stored at -20˚C.

Trypan Blue exclusion viable cell assay

Cells were trypsinized and resuspended in equal volumes of medium and Trypan blue (0.05% solution) and counted using a haemocytometer. Trypan blue dye (Invitrogen, USA) exclusion was used to assess cell viability. Live cells appeared bright because they did not induce trypan blue dye from the membrane, whereas dead cells were observed in dark blue.

XTT cell viability test

Of the MCF-7 and BT-474 cells, $1x10^4$ cells were seeded into 96-well plates and incubated in a 5% CO_2 humidified incubator at 37°C for 24 hours. This allowed the cells to attach to the wells. While TAM was added to the wells at 1 micromolar (µM), 2µM, 5µM, 10µM, 20µM doses [8]. DEBIO 1143 was added at 3µM, 5µM, 10µM, 15µM, 20µM doses. The XTT Cell Viability Test (Biotium, USA) was performed after the 72nd hour. Subsequently 5 mL of the XTT

solution was mixed with 25 µL of the activation reagent. To each well, 100µL activated XTT solution which was half of the total volume (200µL) was added. The cells were incubated in a 5% CO_2 humidified incubator at 37°C for 4 hours. Afterwards, the absorbance value of the wells was measured in the microplate reader (Tecan, Switzerland) at a reference range of 450 - 650 nm. The cell viability was evaluated by CompuSyn 1.0 software.

Synergy determination

Combination index (CI) analysis was performed to determine the synergistic effect of TAM and DEBIO 1143. The proliferative inhibition rate (%) was calculated according to the data obtained from XTT analyses. Data were transformed to fraction affected (Fa; the range 0-1) and was input into CompuSyn software. TAM and DEBIO 1143 synergy quantification was calculated. CI<1, CI=1, and CI>1 values indicated synergism, additive effect, and antagonism in drug combinations, respectively.

Fluorescence microscopy analysis

Fluorescence microscopy analysis was used to investigate cell death. Multi-parameter apoptosis assay kit (CaymanChem, Michigan, USA) was used to show the effect of TAM and DEBIO 1143 combination on MCF-7 and BT-474 cells.

MCF-7 and BT-474 cells were seeded into 6-well plates and incubated in a 5% CO_2 humidified incubator at 37°C for 24 hours. Each well included $1x10^6$ cells. Subsequently, MCF-7 cells were treated with 3.8µM TAM, 10µM DEBIO 1143 and a combination of the TAM and DEBIO 1143; BT-474 cells were treated with 18.9µM TAM, 20µM DEBIO 1143 and a combination of the TAM and DEBIO 1143 in a 5% CO_2 humidified incubator at 37°C for 72 hours. The staining solution was prepared by mixing 2.5µl of tetra methyl rhodamine ethyl ester (TMRE), 2µL of Hoechst stain and 25µL of Annexin V FITC. 250µL of this solution was added into the wells, and 6-well plates were incubated in the dark for 15 minutes. The cells were visualized under a fluorescence microscope (Olympus, Japan).

Statistical analysis

In the experiments, increasing concentrations of TAM and DEBIO 1143 were tested in three wells and each experiment was repeated three times. Dose-response curves for the increasing concentrations of TAM and DEBIO 1143 were generated in Graphpad Prism 5.0 statistical software (La Jolla, CA, USA). Whether there was a significant difference between the controls and the increasing concentrations of TAM and DEBIO 1143 in terms of the percentages changed the cell viability were investigated using the one-way ANOVA and then Dunnett's test. $p<0.05$ values were considered as statistically significant.

Results

XTT cell viability test analysis

TAM (1µM, 2µM, 5µM, 10µM, 20µM) and DEBIO 1143 (3µM, 5µM, 10µM, 15µM, 20µM) by themselves decreased the viability of MCF-7 and BT-474 cells at 72nd hours in a dose-dependent manner($p<0.001$) (Figure 1).

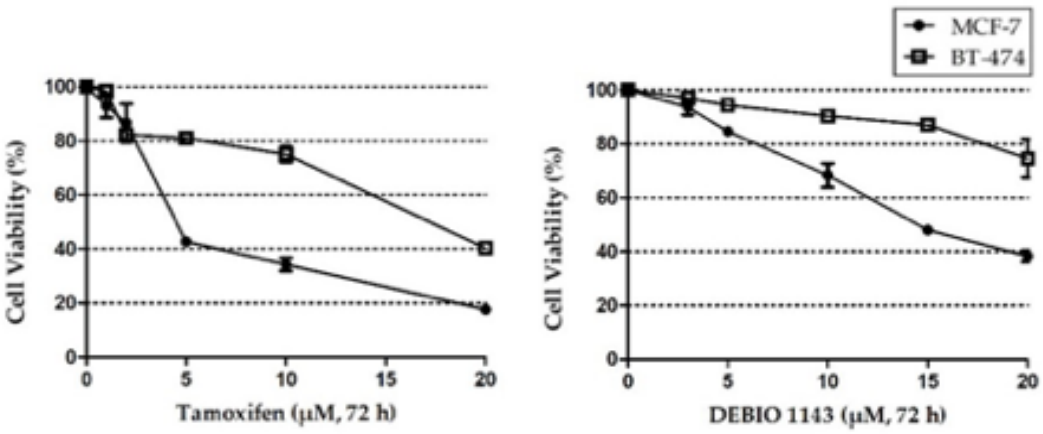

Figure 1. The effect of DEBIO 1143 and tamoxifen on the viability of MCF-7 and BT-474 cell lines at the 72nd hour.

The half maximal inhibitory concentration (IC_{50}) values for TAM at 72nd hours were calculated as 3.8±0.6 μM and 18.9±6.7 μM in MCF-7 and BT-474 cells, respectively, and as 15±0.5 μM in the MCF-7 cell line for DEBIO 1143 (Table 1). No half maximal inhibitory concentration (IC_{50}) value could be obtained for DEBIO 1143 in BT-474 cells.

Data from three different experiments demonstrated that combined doses of TAM and DEBIO 1143 were more effective on MCF-7 and BT-474 cell viability than their single doses (p<0.001). (Figure 2).

The combination index analysis of the combination pairs is summarized in Table 2. Combined doses of TAM and DEBIO 1143 in both cell lines were found to be synergistically effective (CI<1.00).

XTT cell viability data for MCF-7 are given in Table 3 and for BT-474 in Table 4.

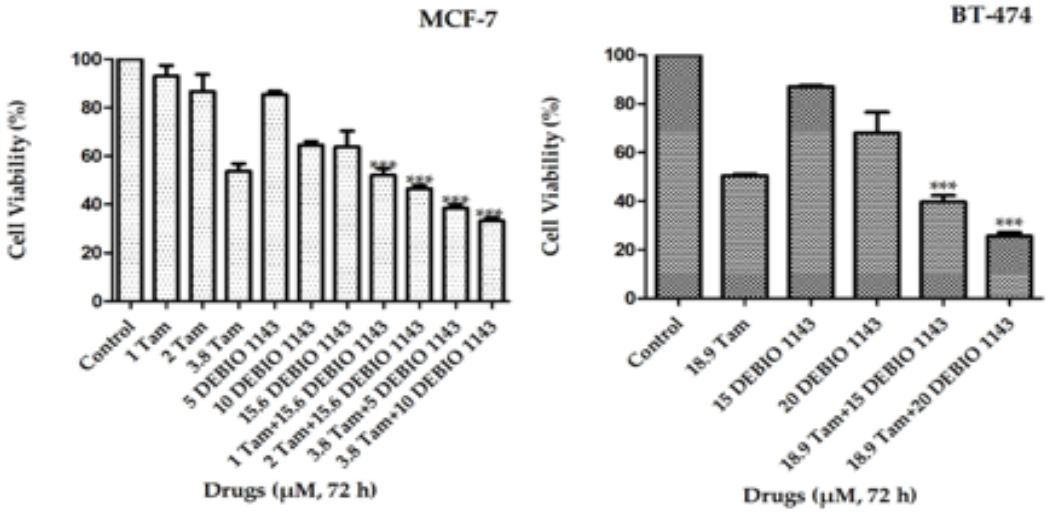

Figure 2. Dose-dependent inhibition of viability of (A) MCF-7 and (B) BT-474 breast cancer cells by tamoxifen, DEBIO 1143 and combination of tamoxifen and DEBIO 1143 (*** p<0.001).

Table 1. IC_{50} values of tamoxifen and DEBIO 1143 on MCF-7 and BT-474 cell lines.

Breast Cancer Cell Line	IC_{50} (μM)	
	Tamoxifen	**DEBIO 1143**
MCF-7	3.8±0.6	15±0.5
BT-474	18.9±6.7	-

IC_{50}: The half maximal inhibitory concentration**; μM:** Micromolar

Table 2. Combination index analysis of DEBIO 1143 combined with tamoxifen in MCF-7 and BT-474 breast cancer cells.

Cell Line	Combined Drugs	Fa	CI*	Meaning
MCF-7	DEBIO 1143 (5 μM) + Tam (3.8 μM)	0.81	0.22	Highly Synergistic
	DEBIO 1143 (10 μM) + Tam (3.8 μM)	0.91	0.20	Highly Synergistic
	DEBIO 1143 (15,6 μM) + Tam (1 μM)	0.52	0.58	Synergistic
	DEBIO 1143 (15,6 μM) + Tam (2 μM)	0.65	0.77	Synergistic
BT-474	DEBIO 1143 (15 μM) + Tam (18.9 μM)	0.85	0.60	Synergistic
	DEBIO 1143 (20 μM) + Tam (18.9 μM)	0.85	0.69	Synergistic

CI: Combination index; **Fa:** Fraction affected

*CI=1.00, CI<0.50, highly synergistic; CI<1.00, synergistic; CI>1.00, antagonistic

Table 3. Statistical *p*-values for the MCF-7 cell line.

Statistical Analysis Findings on MCF-7 Cell line

Compared Groups	*p*-value	Compared Groups	*p*-value
C-$T_{1\mu M}$+$D_{15.6\ \mu M}$	***	$D_{5\ \mu M}$ -$T_{1\ \mu M}$ +$D_{15.6\ \mu M}$	**
C-$T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	***	$D_{5\ \mu M}$ - $T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	***
C-$T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	***	$D_{5\ \mu M}$ - $T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	***
C-$T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	***	$D_{5\ \mu M}$ - $T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	***
$T_{1\ \mu M}$ -$T_{1\ \mu M}$ +$D_{15.6\ \mu M}$	**	$D_{10\ \mu M}$ -$T_{1\ \mu M}$ +$D_{15.6\ \mu M}$	n.s.
$T_{1\ \mu M}$ -$T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	***	$D_{10\ \mu M}$ -$T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	n.s.
$T_{1\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	***	$D_{10\ \mu M}$ - $T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	**
$T_{1\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	***	D_{10}- $_{\mu M}$ $T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	***
$T_{2\ \mu M}$ -$T_{1\ \mu M}$ +$D_{15.6\ \mu M}$	***	$D_{15.6\ \mu M}$ -$T_{1\ \mu M}$ +$D_{15.6\ \mu M}$	n.s.
$T_{2\ \mu M}$ -$T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	***	$D_{15.6\ \mu M}$ -$T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	n.s.
$T_{2\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	***	$D_{15.6\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	n.s.
$T_{2\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	***	$D_{15.6\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	**
$T_{3.8\ \mu M}$ -$T_{1\ \mu M}$ +$D_{15.6\ \mu M}$	n.s.		
$T_{3.8\ \mu M}$ -$T_{2\ \mu M}$ +$D_{15.6\ \mu M}$	n.s.		
$T_{3.8\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{5\ \mu M}$	**		
$T_{3.8\ \mu M}$ -$T_{3.8\ \mu M}$ +$D_{10\ \mu M}$	***		

C: Control; **D:** DEBIO 1143; **T:** Tamoxifen**; n.s.:** non significant

** $p \leq 0.01$; *** $p \leq 0.001$

Table 4. Statistical *p*-values for the BT-474 cell line.

Statistical Analysis Findings on BT-474 Cell line

Compared Groups	*p*-value	Compared Groups	*p*-value
C-$T_{18.9\ \mu M}$ +$D_{15\ \mu M}$	***	$D_{15\ \mu M}$ -$T_{18.9\ \mu M}$ +$D_{15\ \mu M}$	***
C-$T_{18.9\ \mu M}$ +$D_{20\ \mu M}$	***	$D_{15\ \mu M}$ -$T_{18.9\ \mu M}$ +$D_{20\ \mu M}$	***
$T_{18.9\ \mu M}$ -$T_{18.9\ \mu M}$ +$D_{15\ \mu M}$	*	$D_{20\ \mu M}$ - $T_{18.9\ \mu M}$ +$D_{15\ \mu M}$	***
$T_{18.9\ \mu M}$ -$T_{18.9\ \mu M}$ +$D_{20\ \mu M}$	*	$D_{20\ \mu M}$ - $T_{18.9\ \mu M}$ +$D_{20\ \mu M}$	***

C: Control; **D:** DEBIO 1143; **T:** Tamoxifen

* $p \leq 0.05$; *** $p \leq 0.001$

Fluorescence microscopy imaging

Measurement of mitochondrial membrane potential: We determined cell viability using TMRE staining. Subsequently, mitochondrial membrane potential was assessed by a fluorescence microscobe. Healthy mitochondria in the control groups were stained with the red fluorescent stain more than the treated groups. The TMRE staining assay showed that TAM and DEBIO 1143 combination could reduce the viability in MCF-7 and BT-474 cells (Figure 3 and Figure 4).

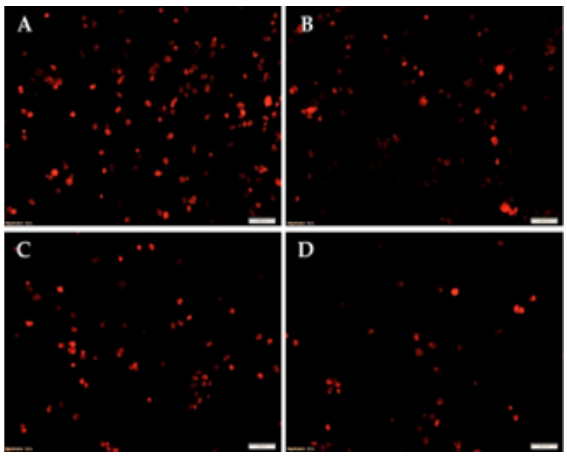

Figure 3. Fluorescent microscope images of MCF-7 cells stained with the TMRE stain (Magnification: 6.3X; Scale: 100 µm). A: Control, B: 3.8 µM tamoxifen, C: 10 µM DEBIO 1143, D: 3.8 µM tamoxifen + 10 µM DEBIO 1143.

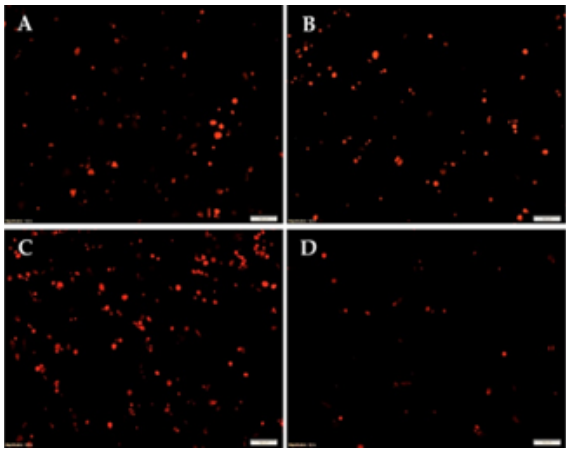

Figure 4. Fluorescent microscope images of BT-474 cells stained with the TMRE stain (Magnification: 6.3X; Scale: 100 µm). A: Control; B: 18.9 µM tamoxifen; C: 20 µM DEBIO 1143; D: 18.9 µM tamoxifen + 20 µM DEBIO 1143.

Evaluation of nuclear morphology: Morphologic differences in nuclei were assessed by a fluorescence microscope after Hoechst staining. Healthy nuclei showed rounded shapes and homogeneous staining. Cells with reduced nuclear size and nuclear fragmentation were suggestive of a decline in cell viability. The Hoechst staining assay demonstrated that TAM and DEBIO 1143 combination could decrease the viability in MCF-7 and BT-474 cells (Figure 5 and Figure 6).

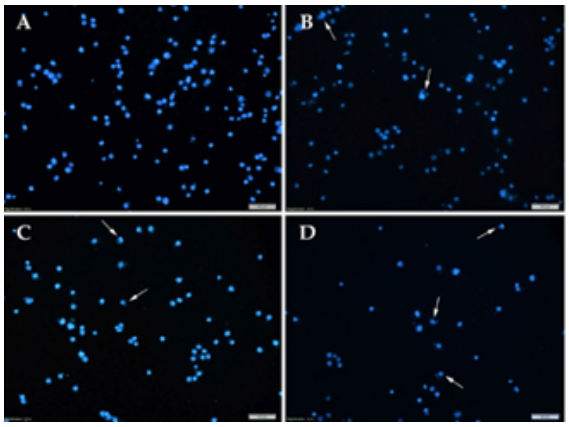

Figure 5. Fluorescent microscope images of MCF-7 cells stained with the Hoechst stain. The arrows B, C, D show the cells with non-robust nucleus (Magnification: 6.3X; Scale: 100 µm). A: Control; B: 3.8 µM tamoxifen; C: 10 µM DEBIO 1143; D: 3.8 µM tamoxifen + 10 µM DEBIO 1143.

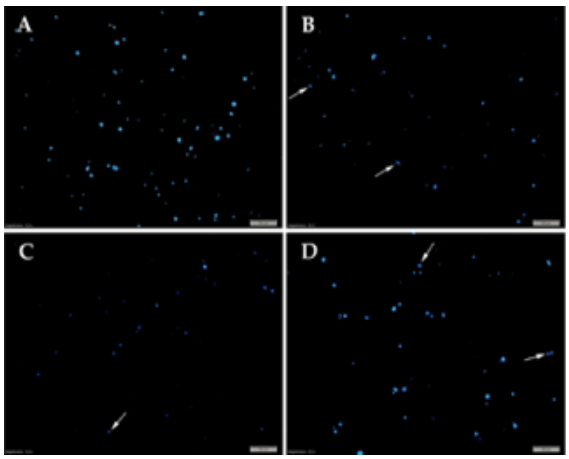

Figure 6. Fluorescent microscope images of BT-474 cells stained with the Hoechst stain. The arrows B, C, D show the cells with non-robust nucleus (Magnification: 6.3X; Scale: 100 µm). A: Control; B: 18.9 µM tamoxifen; C: 20 µM DEBIO 1143; D: 18.9 µM tamoxifen + 20 µM DEBIO 1143.

Phosphatidylserine staining of outer membrane of apoptotic cells: In untreated cell lines and in MCF-7 and BT-474 cell lines treated with TAM and DEBIO 1143 separately or in combination, Annexin V FITC staining, indicator of apoptosis, was not observed.

Discussion

This study is the first to investigate the combined effect of TAM and DEBIO 1143 on MCF-7 (Luminal A type) and BT-474 (Luminal B type) ER (+) breast cancer cell lines. Our findings indicate that the combined administration of TAM and DEBIO 1143 have a synergistic effect in both cell lines. In addition, this effect is not related to apoptosis, perhaps it is due to other cell death types such as autophagy or necrosis.

In the present study, the obtained IC_{50} value of TAM at the 72nd hour was 3.8μM and 18.9μM in the MCF-7 and BT-474 cell lines, respectively. The IC_{50} value in the MCF-7 cell line was similar to the value determined in Wang et al.'s [9] study. The IC_{50} value of TAM at the 72nd hour in MCF-7 cells was 4.12μM in Barett et al.'s [10] study and 10μM in Yenigün et al.'s [11] study. The difference in IC_{50} values may be due to experimental differences such as the number of cells tested, cell multiplication conditions, etc.

In the present study, the IC_{50} value of TAM in the BT-474 cell line at the 72nd hour was higher than that in the MCF-7 cell line. According to the BT-474 cell line gene expression profile, the p53 is mutant and HER2 is overexpressed. In the MCF-7 cell line, p53 is a wild type, and HER2 is not expressed. Overexpression of HER2 is associated with the development of resistance to TAM in breast cancer [12]. In breast cancer cell lines, it is also reported that p53 status affects the cellular response to the estrogen receptor modulator TAM and estrogen ligand, and that p53 mutant cell lines are less susceptible to the cytotoxic effect of TAM, which is considered to result from the fact that the loss of p53 function has led to an increase in cross-talk between the estrogen receptor and the EGFR/HER2 pathway [13]. In the present study, increased IC_{50} value of TAM in BT-474 cell line could be due to the gene expression profile of p53 and HER2.

We obtained 15μM as the IC_{50} value for DEBIO 1143, a SMAC mimetic, in MCF-7 cell line at the 72nd hour. Chessari et al. [14] conducted a study on MDA-MB-231 (ER-, Progesterone (PR)-, HER2-, p53 mutant) and EVSA-T (ER-, PR-, HER2 +, p53 mutant) breast cancer cells. They reported IC_{50} values for DEBIO 1143 at the 72nd hour as 0.019μM and 0.0021μM, respectively. This difference between IC_{50} values may suggest that the mechanism of the cytotoxic effect may be through the hormone receptor profile when the DEBIO 1143 was administered alone. The dose of DEBIO 1143 affecting the ER- and PR- breast cancer cell lines is lower than its dose affecting the ER+, PR+ breast cancer cell lines.

In the XTT cell viability test of the BT-474 cell line, we observed that in the wells which 20μM of DEBIO 1143 was applied, the cell viability was found to be 75%. This is probably due to expression differences in BT-474 cells. Unlike MCF-7 cells, HER2 was overexpressed and P53 was mutant in BT-474 cells, which is may reduce the response against DEBIO 1143. Previous studies have shown that the expression of survivin (a member of IAP family) increases with the overexpression of HER2. p53 can not inhibit the expression of survivin because it is in a mutant state [15]. In addition, it was demonstrated that SMAC mimetics affected IAP levels, and backwards was also true: the high level of IAP affected levels of SMAC. In a study conducted by Ma et al. [16], livin, a member of IAP family, was shown to reduce SMAC.

In this study, the synergistic effect of combined administration on cell viability was observed in MCF-7 and BT-474 cell lines (Table 2). In another study conducted on MCF-7 (ER+, PR+ HER2-), MDA-MB-453 (ER-, PR-, HER2+) and MDA-MB-468 (ER-, PR-, HER2-) breast cancer cells, Fandy et al. [17] showed that treatment with a SMAC mimetic increased the effect of TAM. Consistent with these findings, we found that the combination of TAM and DEBIO 1143 had a synergistic effect on MCF-7 and BT-474 breast cancer cells.

Stanculescu et al. [18] found that estrogen increased the expression of cIAP2 in MCF-7 and T47D ER+ breast cancer cells through the activation of nuclear factor κ beta (NFκβ) by tumor necrosis factor alpha (TNFα). In the present study, 15μM DEBIO 1143 did not have a reducing effect on the BT-474 cell viability, which was probably due to the high level of cIAP2 which may have inhibited DEBIO 1143. In BT-474 cells exposed to the combination of

TAM and DEBIO 1143, it is estimated that cells with decreased estrogen levels cannot express more cIAP2 and thus high synergistic effect can be observed. Furthermore, the difference in the effects of DEBIO 1143 on the breast cancer cell lines may be due to the differences in the expression of the IAP protein types in MCF-7 and BT-474 cells. In a study conducted, it was stated that DEBIO 1143 tended to bind to cIAP1 and cIAP2 and had less affinity for XIAP [19]. Therefore, it would be useful to investigate the levels of IAP expression in MCF-7 and BT-474 cells.

In the fluorescence microscopic analysis performed with Hoechst 33342 staining, it was observed the combined administration of TAM and DEBIO 1143 in MCF-7 and BT-474 breast cancer cells led to a significant increase in cells with distorted nucleus morphology when compared to the separate administration of TAM or DEBIO 1143. It was also determined that in MCF-7 and BT-474 breast cancer cells stained with TMRE had significantly less staining in the combination group than the control group. These data were compatible with the XTT cell viability test.

In vitro studies conducted on the issue have shown that administration of DEBIO 1143 together with bortezomib [20] or carboplatin [4] or radiation [21] or JQ1 [22] can induce apoptosis in various cancer cell lines.

Our results of Annexin V FITC apoptosis analysis suggested that the effect of the combined administration in both cell lines was not due to apoptosis. The decrease in cell viability may have occurred by one of the cell death mechanisms other than apoptosis.

Several studies have shown that TAM induces autophagy and apoptosis in ER positive breast cancer cells [23, 24]. However, some other studies suggest that TAM induces characteristic morphological changes in breast cancer cells consistent with apoptosis [25]. It has also been shown that TAM can induce autophagy in retinal photoreceptors, glioblastoma and breast cancer cells [23, 26, 27]. Bursch et al. [23] showed that inhibition of autophagy inhibited TAM-induced cell death in MCF-7 cells. In another study, autophagosome formation in MCF-7 cells exposed to TAM was imaged through dansylcadaverine, a fluorescent probe of autophagosome [28]. Hwang et al. [29] found that autophagy was induced independent of ER as light chain-3-II (LC3-II) increased in ER+ MCF-7 and ER-SKBR-3 breast cancer cells which were exposed to TAM [26].

In many cancer types, cancer cells escaping apoptosis is a characteristic feature. It is also associated with resistance to treatment. In recent years, the therapeutic effect of necroptosis in cancer cells has drawn attention since it is an alternative mechanism of cell death [30]. Necroptosis is a critical cell death mechanism in response to stress and blocked apoptosis. Necroptosis can be induced by chemotherapeutic drugs. The clinically developed SMAC mimetics trigger necroptosis in addition to apoptosis [31].

Cancer cells with defective apoptotic pathways tend to undergo necroptosis. For example, it has been observed that SMAC mimetics increase TNFα-induced necroptosis in caspase-8 or non-Fas-associated protein with death domain (FADD) leukemia cells [30, 32]. It was also shown that administration of SMAC mimetics together with glucocorticoids induced apoptosis in leukemia cells [33] and necroptosis in leukemia cells without a specific caspase-8 expression [34]. The data accessed by Hannes et al. [35] which were consistent with these findings showed that SMAC mimetics induced necroptosis if caspase activation in pancreatic cancer cells is inhibited. The multifunctional role of receptor-interacting protein 1 (RIP1) should also be considered in response to cellular stress. In cases of cellular stress, if there is no functional caspase-8 or if RIP1 is not ubiquitinated, the cell undergoes necroptosis [36]. In a study conducted on MDA-MB-231 and MCF-7 breast cancer cells, it was thought that RIP1 accumulated due to the destruction of cIAP by a SMAC mimetic, and therefore necroptosis occurred [37].

In the light of our and previous results on the cytotoxic effect of TAM and DEBIO 1143, further studies should be designed to determine whether the effect obtained from the present study was due to necropsy or to autophagy and which agent promotes which death type. This approach will elucidate the mechanisms of synergistic action of TAM and DEBIO 1143. Therefore, we assume that caspase-8, LC3-II, and RIP1 gene expression levels should be

determined in cell lines exposed to TAM and SMAC mimetics.

In conclusion, we determined that the administration of DEBIO 1143 in combination with TAM to ER+ breast cancer cell lines ended in a synergistic effect. Our results show that cell death due to the administration of the drug and a SMAC mimetic alone or in combination was not associated with apoptosis. It is recommended that the expression levels of key proteins associated with apoptosis, necroptosis, and autophagy should be assessed to determine the type of cell death which the effect was associated with. The results of this study, which was conducted on the TAM and DEBIO 1143 co-administered MCF-7 (Luminal A type) and BT-474 (Luminal B type) ER+ breast cancer cell lines, are expected to provide an insight for further pertinent studies.

Conflict of interest: The authors declare no conflicts of interest.

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Compliance with ethics guidelines

This article does not contain any studies with human or animal subjects performed by any of the authors.