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In Vitro Evaluation of Cytotoxic and Antitumor Activities of The Tamoxifen and Doxorubicin Combination on MCF-7 and BT-474 Breast Cancer Cell Lines

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Highlights:

- Alternative treatment strategy for breast cancer
- Drug combination analysis on breast cancer cell lines
- qPCR analysis of *Bcl-2, Mapt, Mrp1* genes

Keywords:

- Combinationtherapy anticancer drug
- *Bcl-2*
- *Mapt*
- *Mrp1*

ABSTRACT:

The combination therapy of breast cancer has preferred for the patients to minimize possible side effects, drug resistance, recurrence and toxic effects. In this study, we aim to investigate the cytotoxic and antitumor activities the tamoxifen and doxorubicin combination in breast cancer cell lines, MCF-7 and BT-474. Tamoxifen (Tam) and doxorubicin (Dox) and their combination with different concentrations (0.625–20 µM Tam; 0.0625–2 µM Dox and 5 µM Tam+ 0.5/1.0/1.5 µM Dox combination were applied to MCF-7 and BT-474 cells for 48 hours. Afterthat, their cytotoxic activities were analyzed with MTT assay. *Bcl-2*, *Mapt* and *Mrp1* are genes that induce cell proliferation, inhibit apoptosis and play role in drug resistance in cancer cells. To evaluate the antitumor activities of these genes in combination treatment, mRNA levels were analyzed by quantitative PCR. According to the MTT assay, it was determined that IC_{50} values as 17.26 µM and 16.65 µM for tamoxifen on MCF-7 and BT-474 breast cancer cell lines. IC_{50} values of doxorubicin in MCF-7 and BT-474 cells were 1.65 µM and 1.57 µM, respectively. It was found that the application of the combination drugs (15 µM tamoxifen and 1.5 µM doxorubicin) in MCF-7 and BT-474 cells have the lowest combination index values as 1.09 and 1.26, respectively. Therefore, the combination of 15 µM tamoxifen and 1.5 µM doxorubicin was selected and applied to both breast cancer cell lines for gene expression analysis. It was found that while *Mrp1* and *Mapt* genes expressions were significantly upregulated, *Bcl-2* gene expression was downregulated in MCF-7 cells. However, *Bcl-2*, *Mrp1* and *Mapt* genes expressions in BT-474 cells were not significantly regulated. Altogether, these findings suggest that the combination of these two drugs may have a potential antagonistic interaction according combination index values.

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INTRODUCTION

Doxorubicin (Dox) is an effective chemotherapeutic drug and anthracycline type antibiotic used for treatment of metastatic breast cancer (BC) (Thomadaki &Scorilas, 2008). Tamoxifen (Tam) is also drug that preferred to treat estrogen receptor positive BCs including postmenopausal women of metastatic sites (Zheng et al., 2007). However, long term usage of of them may lead to serious side effects. For example, as the doxorubicin causes the cardiotoxicity, it could be preferred to use with adjuvants. Besides, tamoxifen leads to hot flushes, vaginal discharge and vaginal dryness. Multi-drug treatments such as combination of Tam and Dox may be more effective in the treatment of BC, rather than long-term treatment with a single drug (Sheppard et al., 1977). It has been shown that doxorubicin combination with tamoxifen may be a good choice after relapse in BC patients (Das et al., 2022).

The antitumor activity of both doxorubicin and tamoxifen has been associated the drug resistance. The difference on the regulation of drug transporter proteins such as P-glycoprotein, members of the multidrug resistance protein (MRP1) may activate drug resistance mechanism (Müller et al., 1998; Borst et al., 2000; Dallavalle et al., 2020). It was reported that MRP1 protein expressions were higher in doxorubicin drug-treated MCF-7 cell lines than in untreated cell (Kocdogan et al., 2020). B-cell lymphoma 2 gene (*Bcl-2*) could have been considered as antiapoptotic and drug resistance marker in cancers. Furthermore, chemotherapeutic agents could result in apoptosis through inhibiting overexpression of *Bcl-2* gene (Seong et al., 2015). In tamoxifen treated MCF-7 cells, *Bcl-2* was downregulated that activates Tp53 signaling during apoptosis (Sheppard et al., 1974). Microtubule-associated protein tau (MAPT) shows sensitivity to the some taxel group drugs in BC (Rodrigues-Ferreira et al., 2019). Therefore, the low level of expression of MAPT could creat antitumor activity for BC cells (Drechsel et al., 1992; Park et al., 2007).

Several studies have demostrated that there are different results about the effect of Tam/Dox combination in human BC cells. For example, while Chuang et al. (2013) reported that combination of tamoxifen and doxorubicin had synergistic effect on the inhibition of proliferation BT-483, Woods et al. (1994) reported that the combined drug of Tam/Dox combination had antagonistic interaction and did not significantly change the antitumor effects of the cells (Woods et al., 1994; Chuang et al., 2013). In this study, firstly, we aimed to determine the cytotoxic activities of the tamoxifen and doxorubicin in both MCF-7 and BT-474 cells. After the determination of the concentration Tam/Dox combination, antitumor activities of Tam/Dox combination in MCF-7 and BT-474 cells were determined by quantitative PCR (qPCR) assay.

MATERIALS AND METHODS

Drugs

Dox was purchased as vials from Adooq Bioscience (California; USA, cat. No: A14403). Dox hydrochloride was soluble in DMSO. Tam was obtained from MedChemExpress (New Jersey; USA, cat. No: ICI 47699) and was also solved in DMSO.

Cell culture

Michigan Cancer Foundation-7 (MCF-7) and BT-474 cells in this study were **given by Dr. Bala**. MCF-7 cells were grown in DMEM/ high glucose (Capricorn Scientific GmbH, Germany) including 10% FBS and %1 pen/strep and 2 mM L-glutamin. However, BT-474 cells were grown in RPMI media (Capricorn Scientific GmbH, Germany). All cells were cultured in a 5% CO_2, 37 °C incubator. In experiment set, the cells were grouped into four; the control group was treated with 0.5% DMSO in

DMEM/RPMI medium; the two cell groups were treated with Dox (0.5/1.0/1.5 μM) or Tam (10-15 μM) and their combination.

Cytotoxicity analysis

The cell viability was processed by using the MTT (USA, Sigma cat. No: M5655) method described by Kazan's group (Kazan et al., 2020). Different concentrations of the drugs as the following ranges: tamoxifen (0.625–20 μM); dox (0.0625–2 μM); tam + dox in different concentrations with combination (15 μM Tam + 0.5/1.0/1.5 μM Dox concentrations and 10 μM Tam + 0.5/1.0/1.5 μM Dox) were applied to both cell lines. After 48 hours, MTT test was performed. Each plate was read spectrophotometrically (Evolution-201/Thermo Scientific /USA) at the wavelength of 570 nm. While the control group cells were assumed viable as 100%, viable cells ratio of other groups were calculated relatively according to absorbance values of them to control groups. The determination of IC_{50} values were processed by the help of GraphPad Prism 8.0 software. The effect of combination of drugs as Combination index (CI) was calculated according to Chou et al. method (Chou & Talalay, 1984).

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the cells using a Ribospin II (Korea, GeneAll cat. No: 314-150) RNA isolation kit. In order to synthesize complementary DNA (cDNA), A.B.T. KİT P protocol was performed (Turkey, A.B.T. cat. No: C03-01-05). The reaction was set with *Bcl-2, Mapt and Mrp1* primers as shown and cited in Table 1 and mix with SYBR Green Mastermix (Turkey, A.B.T. cat. No: Q03-02-01) using Biorad CFX96 qPCR system. The fold change of each gene was calculated using comparative threshold cycle (C_t) method as normalized to that of *Gapdh* ($2^{-\Delta\Delta Ct}$). All experiment reactions were done in triplicate.

Table 1. Primer Sequences for qPCR Analysis

Primer	Forward 5'----->3'	Reverse 5'----->3'
Bcl-2 (Boyle et. al., 2015)	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
Mapt (Young, 2021)	TAGGCAACATCCATCATAAACCA	TCGACTGGACTCTGTCCTTGAA
Mrp1 (Gao et. al., 2016)	GTCGGGGCATATTCCTGGC	GGGCATCCTCTTTTAAGGCTG
Gapdh (universal)	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT

Statistical data analysis

All experiments were replicated in triplicate. Means of replicates and their standard deviations were calculated. Data normality was done using IBM SPSS 22.0 program.

RESULTS AND DISCUSSION

The Cytotoxicity Results

The viability assay is usually performed measuring the toxic effect of a drug which can be determined the half maximal inhibitory concentration (IC_{50}) value (AL-Jailawi et al., 2015). When the IC_{50} values of Tam in the MTT assay was analyzed, MCF-7 and BT-474 cells have 17.26 μM and 16.65 μM, respectively (Figure 1A). However, the IC_{50} values of Dox in two cell lines were 1.65 μM and 1.57 μM, respectively (Figure 1B).

According to our results, combination index (CI) was calculated and the combined doxorubicin and tamoxifen effects was determined in terms of having drug synergism (CI < 1), additive effect (CI = 1), or antagonism (CI>1). The cell viability results of MTT assay in both MCF-7 and BT-474 cells were used to calculate CI value of with Dox and Tam. We found that the combined drug effects of Tam and Dox had antagonistic effect in MCF-7 cells because the CI values of all combination treatment were higher than 1 (Table 2). Thus, the antagonist effect was observed along tamoxifen and doxorubicin combination.

Table 2. Combinationİ (CI) Values After Tam/Dox Exposure.

Cell Line	Combination	Combination Index
MCF-7	0.5 µM Dox/15 µM Tam	1.74
	1.0 µM Dox/15 µM Tam	1.83
	1.5 µM Dox/15 µM Tam	1.09
	0.5 µM Dox/10 µM Tam	1.67
	1.0 µM Dox/10 µM Tam	1.54
	1.5 µM Dox/10 µM Tam	1.50
BT-474	0.5 µM Dox/15 µM Tam	1.54
	1.0 µM Dox/15 µM Tam	1.46
	1.5 µM Dox/15 µM Tam	1.26
	0.5 µM Dox/10 µM Tam	1.50
	1.0 µM Dox/10 µM Tam	1.41
	1.5 µM Dox/10 µM Tam	1.46

*The combination index (CI) for two drugs can be defined based on Chou and Talalay (14) median effect equation

The lowest CI values in both MCF-7 cells and BT-474 cells were 1.09 and 1.26, respectively, in the combination of 15 µM Tam and 1.5 µM Dox. Therefore, the combined drug concentration for the gene expression experiments was chosen as 15 µM Tam/1.5 µM Dox. Two drugs with seperately and different concentration combination were cultured for 48 hours incubation. 20 µM tamoxifen treatments on the cells alone decreased the number of viable cells in MCF-7 and BT-474 below 50% **(p< 0.05).** The cells were cultured in 2 µM doxorubicin for 48 hours, the viabilities of MCF-7 and BT-474 cells were 43% and 41%. However, when the doxorubicin concentration was decreased to 1 µM, the viabilities of the MCF-7 and BT-474 were 61%, 59% respectively. The exposure of 10 µM Tam alone to MCF-7 and BT-474 cells, viability ratios of cells were 68% and 65%. Therefore, culturing concentrations of the combination of tamoxifen and doxorubicin was determined based on viability ratios of cells. At all diffferent concentrations of combination of Tam/Dox on the cells, the number of viable cells never vitally decreased. When combined with 1.5 µM Dox, 15 µM Tam could exhibit low cytotoxic effect that decreased the viability of cells to 55%. In addition, it was found that the sensitivity of cells to the other combinations of Tam/Dox did not increase dramatically.

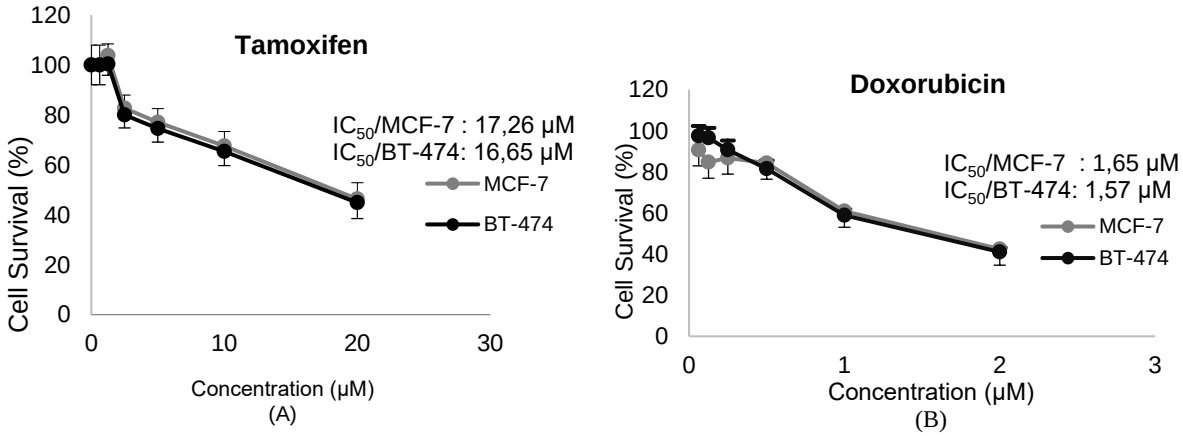

Figure 1. Cell Viability With MTT Assay. Dose Response Curve of Tamoxifen (A), and Doxorubicin (B), for 48 Hours (black and gray diamonds) on MCF-7 and BT-474 cells. All Experiments Were in Triplicate

At concentrations of 15 µM Tam/1.5 µM Dox, the ratio of the viable cells did not essentially change at both the combination of Tam/Dox and Dox alone (1.5 µM Dox).

Figure 2 showed that the culturing of BT-474 cells at any of combinations of Tam/Dox did not decrease of the growing of the cells while Tam/Dox combination had a slight effect on cell proliferation of MCF-7 cells. It was observed that the drugs were antagonistic to each other because the treatment of the drugs alone was more effective on decreasing of the viable cells.

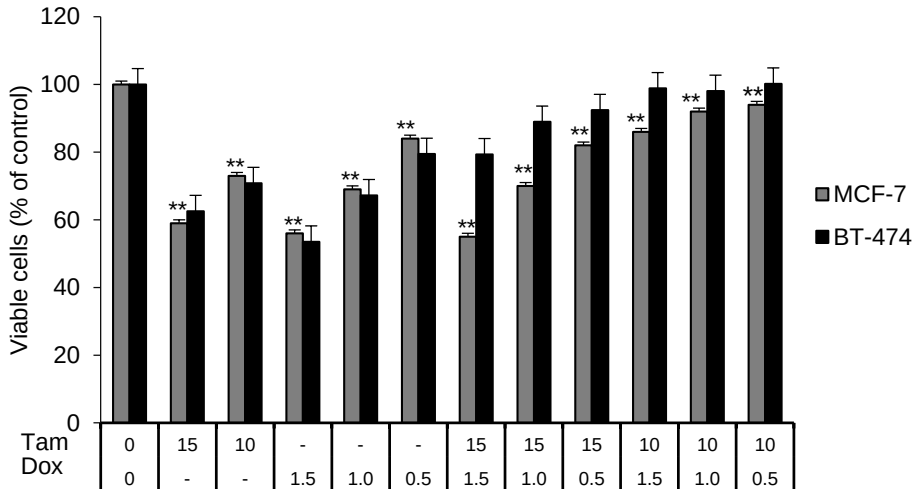

Figure 2. The Viability Ratios of Two Cell Lines Exposed to the Combination of Tamoxifen (Tam) and Doxorubicin (Dox) (**p<0.01)

In previous studies, it has been reported that tamoxifen reduced the number of viable MCF-7 cells by becoming toxic or apoptotic effect (Ajabnoor et al., 2012). It has been previously documented that tamoxifen targeted the disruption of cell membrane integrity by altering in the compositon of plasma membrane (Salgueiro et al., 2014; Khadka et al., 2015). It has been also shown that IC50 value of tamoxifen in MCF-7 and BT-474 cells were 3.2 μM and 5.7 μM, respectively (Pawlik et al, 2016). Besides, IC50 values of doxorubicin in MCF-7 and BT-474 cells were 0.69 μM and 1.14 μM, respectively (Wen et al., 2018). Our results were supported, which is consistent with previous literature.

Ouyang and Li reported that cell proliferation of MG63 was inhibited by Tam and Dox alone. They also found that the combination of Tam and Dox had significantly antagonistic effect on MG63 cell lines (Ouyang & Li, 2013). Ahmann et al. (1985) found the synergistic effect of Tam, Dox and cyclophamide combination in clinical patients with ER+positive tumors (Ahmann et al., 1985). In another study, the combination of Tam/Dox with radiotherapy had relapsed the improvement of tumors in ER+ patients (Blomqvist et al, 1992).

QPCR results of *Bcl-2, Mapt, Mrp1* genes

The treatment of combined doxorubicin and tamoxifen changed the mRNA expression levels of *Bcl-2, Mapt, Mrp1* genes in MCF-7 cells. In our study, qPCR analysis of the expression of *Bcl-2, Mapt, Mrp1* genes was processed in order to figure out the antogonistic effect between Dox and Tam. The mRNA expression level of *Bcl-2, Mapt, Mrp1* genes were analysed by qPCR.

In our study, the mRNA gene expression levels of *Bcl-2* statistically significantly decreased 2.4 fold in MCF-7 for Tam/Dox combination (Table 3). Interstingly, MCF-7 cells were exposed to 15 μM Tam and 1.5 μM Dox alone, only Tam were caused statistically significant 1.2 fold decrease in *Bcl-2* expression. However, when both were applied together (15 μM Tam + 1.5 μM Dox), it was determined that there was a statistically significant 2.4-fold decrease in *Bcl-2* expression. However, BT-474 cell lines did not show same pattern. Although *Bcl-2* gene expression was not statistically significantly decrease (0.85 fold) in Tam/Dox combination, on the contrary, down regulation was not increased compared to the 15 μM Tam (1.1 fold) and 1.5 μM Dox (1.0 fold) alone application.

Table 3. qPCR Results of *Bcl-2* on Two Cell lines. Treatment with 15 µM tamoxifen (Tam)/1.5 µM doxorubicin (Dox) combination compared to their control group cell lines without exposure to drugs and incubated for 48 hours. **(↓: downregulation)** (*p<0.05)

Drug Concentration	MCF-7 Fold Change	BT-474 Fold change
15 µM Tam	↓1.2*	*↓1.1*
1.5 µM Dox	↓0.8	↓1.0*
15 µM Tam+ 1.5 µM Dox	↓2.4*	↓0.85

In this study, it was found that *Mapt* gene expression levels showed 1.2, 1.1 and 1.44 fold statistically significant upregulation in MCF-7 cells in the presence of tamoxifen, doxorubicin and Tam/Dox, respectively (Figure 3). On the other hand, when BT-474 cells treated in a similar way, *Mapt* expression did not show statistically significant upregulation.

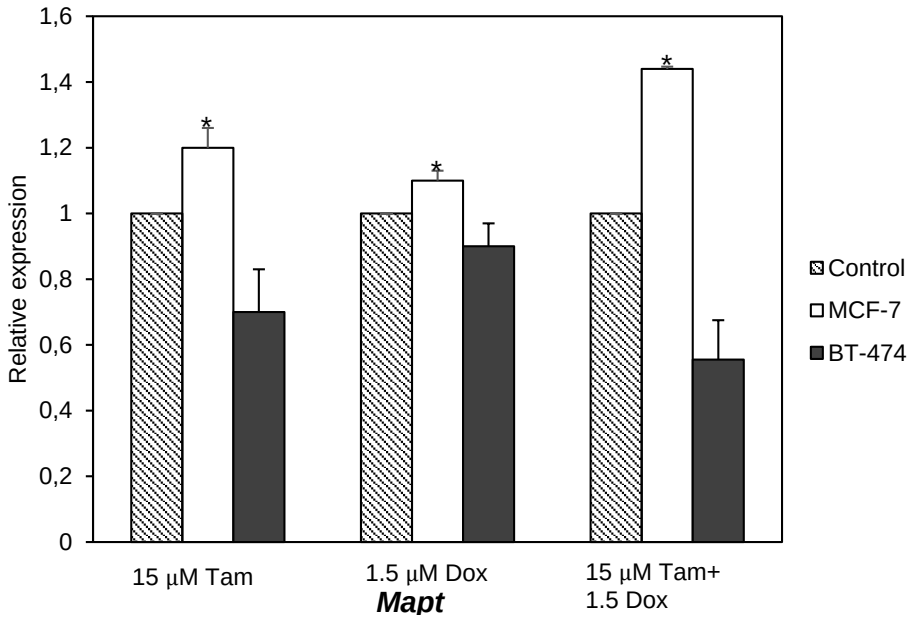

Figure 3. qPCR results of relative mRNA levels of *Mapt* on two cell lines. Treatment with 15 µM tamoxifen (Tam)/1.5 µM doxorubicin (Dox) combination compared to their control group cell lines without exposure to drugs and incubated for 48 hours (*p<0.05)

In our study, it was found 1.46 and 1.2 fold statistically increase in *Mrp 1* gene expression in MCF7 and BT-474 cells, respectively, when 1.5 µM doxorubicin treated to cultures. Similar to doxorubicin, 15 µM tamoxifen treatment caused statistically increase in *Mrp1* gene expression 2.6 and 2.23 fold in MCF-7 and BT-474 cells, respectively. According to our results, Tam/Dox treatment caused 3,8 fold statistically increase in *Mrp1* gene expression level in MCF-7 cells, whereas it did not change in BT-474 cells.

The higher upregulation of *Mrp1* gene expression was found than treated with Dox as the MCF-7 and BT-474 cells were treated with Tam. Furthermore, it was determined that there was a significant increase the mRNA level of *Mrp1* gene in MCF-7 cells treated by Tam/Dox combination, which could not be determined the similar increase expression of *Mrp1* gene for BT-474 cells. As a result, it could be concluded that *Mrp1* expression was associated with both anthracycline and endocrine therapy. It is also suggested that *Mrp1* activation and *Bcl-2* inactivation probably contributes to decrease of viability of MCF-7 cells by the exposure of Tam/Dox.

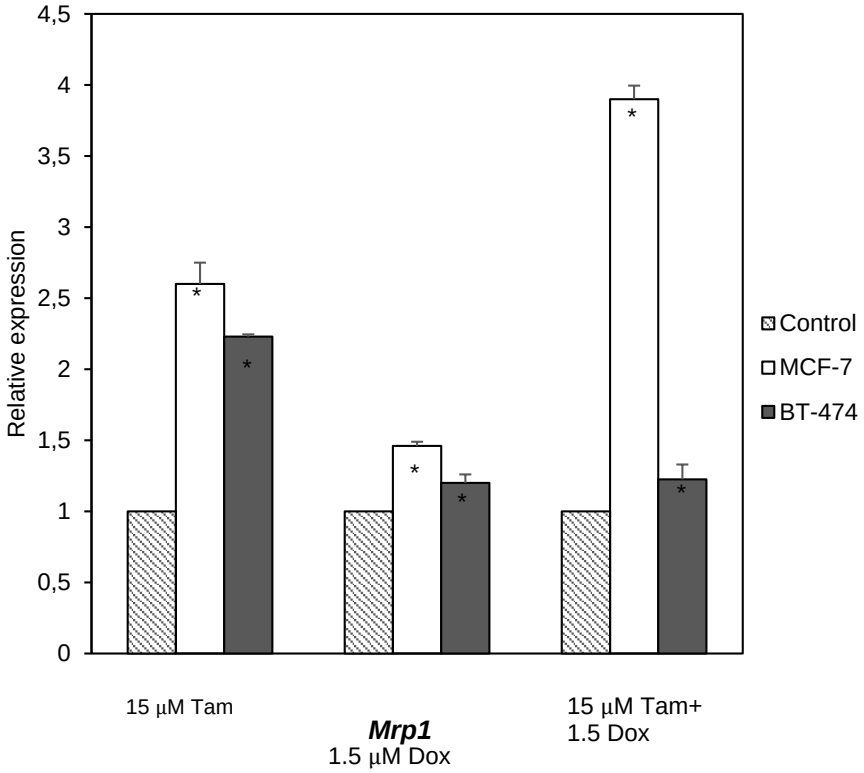

Figure 4. qPCR results of relative mRNA levels of *Mrp1* on two cell lines. (*p<0.05)

The results indicated that, while the mRNA expression levels of *Bcl-2* gene were downregulated, *Mapt* expression did not change vitally and *Mrp1* expression was upregulated significantly when MCF-7 cells exposed to Tam/Dox combination. However, it was not determined any regulation of those genes in BT-474 cells. We compared the mRNA profiles *Bcl-2, Mapt, Mrp1* genes that exposure of Tam, Dox, Tam/Dox treatment to MCF-7 and BT-474 cells. According to our results, it was pointed out as follows: (a) the combination of tamoxifen and doxorubicin may contribute to antagonism according to Combination index values (b) the mRNA levels of *Bcl-2* were statistically significantly downregulated in the treatment of the combination of Tam/Dox in MCF-7 cells (c) the mRNA levels of *Mapt* gene was upregulated statistically significantly in MCF-7 cells (d) the levels of *Mrp1* mRNA were statistically significant increase in MCF-7 and BT474-cells.

Apoptosis steming from Dox activates the Bcl-2 family of proteins activating caspases was shown by different studies (Rossé et al., 1998; Zhang et al. 1999; Hawkin et al, 2000; Salami & Karami-Tehrani, 2003). However, it was shown that tamoxifen regulates the apoptotic changes in cells by downregulating of Bcl-2 expression in cells. Apoptosis induced by chemotherapeutic drugs has been determined through down regulation of Bcl-2. The decreasing of Bcl-2 expression in our expreiment is reasonably consistent with related literature. In additon, the upregulation of Bcl-2 expression is related tumor growth (Kocdogan et al., 2020). Mapt expression was positively correlated with ER expression and ER signaling has an effect on Mapt expression (Andre et al., 2007). Similar to our MCF- 7 results, a research team observed that protein expression of MAPT was increased by exposure with tamoxifen (Ikeda et al, 2015). In addition, Rouzier et al. found that paclitaxel and 5-fluorouracil, doxorubicin, cyclophosphamide drug combination resulted in down regulation of Mapt expression (Rouzier et al., 2005). Chemotherapeutic drugs could be exported from cancer cells by Mrp1 whose expression is upregulated (Kim, 2015). The regulation of Mrp1 gene expression has become differently in various cells including BC cells. The overexpression of Mrp1 is the proof of the resistance of different cancer cells to chemotherapy drugs

(Wind & Holen, 2011). Wang et al. reported that the Mrp1 gene expression had higher regulated in MCF-7/Dox cells than that in MCF-7/Docetaxel and MCF-7/wild type (Wang et al., 2014).

CONCLUSION

The combination treatment of Tam/Dox caused antagonistic effect on MCF-7 and BT-474 cancer cell lines, thus the number of tested cells reduce by more than 40%. This combination regimen (15 μM Tam/ 1.5 μM Dox) exposed to both MCF-7 and BT-474 cell lines resulted in the increase of mRNA expression of *Mrp1* and decrease of *Bcl-2* gene expression. It is considered that the antitumor properties of the cells might be regulated by these two genes. It was determined no correlation in expression level of *Mapt* gene in MCF-7 cells after treating with Tam/ Dox. Our study may be a candidate to be one of the current approaches to use various drugs together with anti-cancer drugs. Tam/Dox combination regiment for ER+ breast cancer could be applied in futher studies related with breast cancer studies.

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Conflict of Interest

The author declared that they have no conflict of interest and all authors have read and approved of the manuscript being submitted.

Author's Contributions

Menderes SUICMEZ and Gamze NAMALIR prepared the manuscript's backbone and wrote the original draft of the manuscript. Hilal OZDIL helps to the other authors about conducting experiments."

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