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# *In vitro* apoptotic and antiproliferative effects of propranolol on human breast cancer cells

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Breast cancer is an issue of concern with increasing incidence among women worldwide. Propranolol, as an antihypertensive drug, exerts anticancer effects too. We conducted this study to analyze the *in vitro* apoptotic and anti-proliferative effects of propranolol in human MCF-7 breast cancer cells. MCF-7 cells were seeded into 6-well plates and treated with 50 µL propranolol for 24 hours. After cell homogenization, the levels of pro-apoptotic proteins BCL2 associated X (BAX), apoptosis inducing factor (AIF), C/-EBP homologous protein (GADD153), and glucose-regulated protein 78 (GRP78), anti-apoptotic protein BCL2 apoptosis regulator (BCL-2), and cycle-regulator WEE1 G2 checkpoint kinase (WEE1) were measured with ELISA. Propranolol significantly upregulated pro-apoptotic proteins AIF, BAX, GADD153, and GRP78 while downregulated anti-apoptotic protein BCL2. The level of WEE1, as the main regulatory cell cycle protein at the G2/M checkpoint, significantly increased after propranolol treatment. Propranolol inhibited the proliferation of MCF-7 human breast cancer cells by upregulating pro-apoptotic factors AIF, BAX, GADD153 and GRP78 and by downregulating antiapoptotic BCL2. Elevated WEE1 levels after propranolol treatment might lead the tumor cells into a sustained cell-cycle arrest which eventually resulted in caspase-dependent or -independent mitochondrial or endoplasmic-reticulum stress-induced apoptosis. So, propranolol can be utilized as a potential therapeutic agent in breast cancer therapy.

**Keywords**: Anitcancer, Antitumorigenic activity, MCF7

Breast cancer which had become a serious issue with an elevating incidence holds the leading share with 32% of new cancer diagnoses and is the second deadliest cancer type after lung cancer among women in US, 2024.[1] Non-melanoma breast cancer types like squamous breast cell carcinoma are one of the most prevalent malignancies in the US with an incidence of up to 1% of all breast cancer cases[2]. The most challenging dimension of cancer therapy is the relapse of cancer after remission or complete removal of the tumor[3]. β-adrenergic receptor antagonists, so-called β-blockers, can exert potential beneficial effects as an adjuvant therapeutic option in the treatment of cancers by inhibiting metastasis, tissue invasion, and angiogenesis[4]. Continual adrenergic signalization, overexpression of DUSP1 via noradrenalin, hypothalamic-pituitary-adrenal axis, and upregulated levels of PGE2 as a response to cancer-related stress are among the relevant pathophysio-logical mechanisms of tumorigenesis and β-blockers[5,6]. In a study conducted with hypertensive patients, it was claimed that daily $Ca^{2+}$ channel-blocker medication which eventually blocks the L-type $Ca^{2+}$ channels on the cell membrane might be a risk factor for the tumorigenesis[7]. A similar elevated risk for tumorigenesis and $Ca^{2+}$ channel-blocker was also found in another study[8]. A previous study with prostatic glandular cells showed that the elevated intracellular free $Ca^{2+}$ levels triggered cellular apoptosis[9]. However, the sound evidence in previous *in vitro* studies demonstrated that increased cellular $Ca^{2+}$ level is not a universal prerequisite to promote apoptosis but rather impaired $Ca^{2+}$ homeostasis is required to induce apoptotic pathway[10,11].

Apoptosis is a key mechanism in the eradication of cancer cells[12]. Beside the effective conventional combinatory treatment strategies in breast cancer patients,[13,14] the alternative treatment agents against chemo-resistant cancer types bear the potential with

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their apoptosis-triggering activity[15]. β-blockers have the potential as an adjuvant agent in cancer treatment to reduce resistance against chemotherapy[4]. Thus, propranolol, as a non-selective prominent β-blocker, can be a promising therapeutic agent against breast cancer cells by inducing apoptosis. In this context, here, we investigated the *in vitro* apoptotic and antiproliferative effects of propranolol in human MCF-7 breast cancer cells. For this purpose, prominent pro-apoptotic proteins BCL2 associated X (BAX), apoptosis inducing factor (AIF), C/-EBP homologous protein (GADD153), and glucose-regulated protein 78 (GRP78) and antiapoptotic protein BCL2 apoptosis regulator (BCL-2) as well as cell cycle-regulatory protein WEE1 G2 checkpoint kinase (WEE1) were selected to analyze the effect of propranolol on the human breast cancer cells[16].

## Materials and Methods

### Cell culture

Human breast cancer cell line MCF-7 was obtained from the cell culture stock of the Cukurova Pharmacology laboratory. The cells were cultured as described previously[17]. Shortly, the cells were propagated in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 1% L-Glutamine (Hyclone), 10% Fetal Bovine Serum (FBS, Hyclone), and 1% Penicillin/Streptomycin (Hyclone) medium in T75 cell culture flasks (Corning). The cells were incubated under 37°C and 5% $CO_2$ conditions for proliferation. After cells reached 70-80% confluence, they were passaged.

### Cell homogenization and protein isolation

The protein isolation following homogenization was performed as described previously with minor modifications[18]. Briefly, the cells were seeded into 6-well plates at a ratio of $1\times10^5$ cells/well. After the cells reached confluence, 50 µM propranolol (Sanofi Aventis, Istanbul, Turkey), as sub-$IC_{50}$ effective concentration[19], was added into the culture medium and incubated for 24 h in a $CO_2$ incubator. After that, the cells were washed with ice-cold PBS (Sigma-Aldrich) once and incubated in RIPA buffer (Sigma-Aldrich) for 15 min. on ice. Then cell lysate was collected with a cell scraper and centrifuged at 15000 rpm for 20 min and supernatants were transferred into microfuge tubes and kept at −20°C. All protein samples were quantified with Bradford assay using bovine serum albumin standard solution and Bradford reagent (Bio-Rad Laboratories). Total protein concentrations were used for the standardization of the ELISA tests.

### Enzyme-linked immunosorbent assay (ELISA)

In order to analyze protein levels of apoptosis inducing factor mitochondria associated 1 (AIF), BCL2 associated X (BAX), apoptosis regulator (BCL2), DNA damage inducible transcript 3, (GADD153, also known as DDIT3), heat shock protein family A (Hsp70) member 5 (GRP78), and G2 checkpoint kinase (WEE1), commercial ELISA kits were purchased from "Shanghai Sunred Biological Technology Co., Ltd" and the ELISA experiments were performed according to manufacturer's protocols. Optical densities were detected spectro-photometrically (Thermo, Multiskan, Finland) and relative concentrations were calculated based on standards.

### Statistical analysis

All the experimental data were analyzed to check the Gaussian distribution using the Shapiro–Wilk test. In the comparison of normally distributed data in two individual groups, the parametric unpaired t-test was performed. The experiments were performed in triplicate. All data were expressed as mean ± SD. $P$ <0.05 value was accepted as statistically significant.

## Results

In this study, we analyzed apoptosis and cell-cycle regulatory proteins with ELISA after treatment of MCF-7 cells with 50 µM propranolol for 24-h. ELISA data revealed that protein levels of pro-apoptotic markers AIF, BAX, GADD153, and GRP78 were significantly upregulated while BCL2 level, as an anti-apoptotic regulator, decreased after propranolol treatment in MCF-7 cells (Fig. 1 A-E, $P$ <0.05).

We also measured the level of cell-cycle control protein WEE1 to check the status of proliferating tumor cells. We found a significant increase in WEE1 level after propranolol treatment in MCF-7 cells (Fig. 1 F, $P$ <0.05).

## Discussion

We analysed the effect of a non-selective β-blocker drug called propranolol, commonly used against hypertension in patients, on the regulatory proteins involved in both apoptosis and cell cycle control in human breast cancer cell line MCF-7 with the ELISA method. In an *in vivo* ovarian cancer model, the propranolol treatment could reverse the tumor angiogenesis induced by β-adrenergic activation by

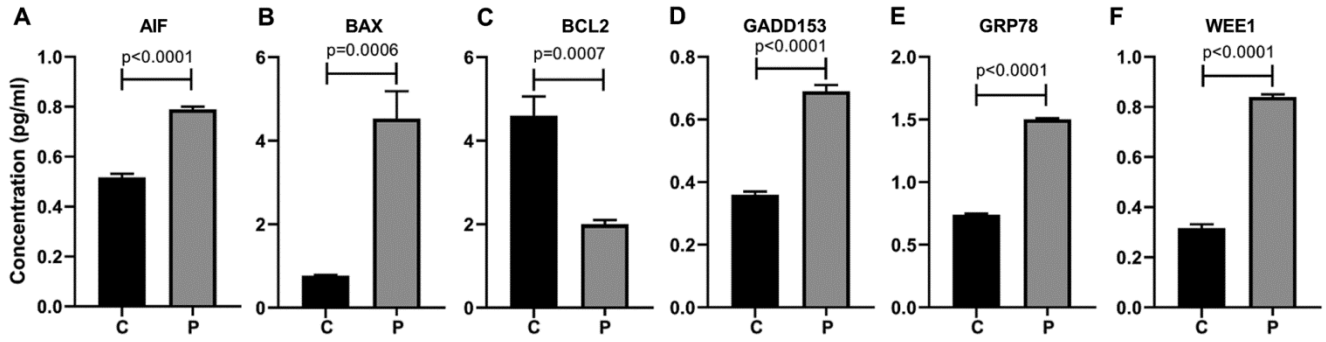


Fig. 1 — Effect of propranolol treatment on protein levels (pg/mL) of (A) AIF; (B) BAX; (C) BCL2; (D) GADD153; (E) GRP78; and (F) WEE1 assessed with ELISA method after 50 μM propranolol treatment for 24 h in MCF7 human breast cancer cells. [All data were depicted as mea±SD. Unpaired t-test was used to compare propranolol and untreated control groups. $n$=3, $P$ <0.05 was accepted as significant. BAX: BCL2-associated X, AIF: Apoptosis inducing factor (AIF), GADD153: C/-EBP homologous protein, GRP78: glucose-regulated protein 78, BCL2: BCL2 apoptosis regulator WEE1: WEE1 G2 checkpoint kinase. SD: Standard deviation]

blocking the cAMP–PKA signaling pathway[20]. Propranolol can exert its anti-tumorigenic effects via the blockade of angiogenesis besides triggering apoptosis and inhibiting the proliferation of tumor cells. In another *in vitro* pancreatic cancer model, it was demonstrated that propranolol could suppress cell proliferation via intrinsic apoptosis[21]. Another study conducted with various pancreatic cancer cell lines claimed that propranolol could block the invasion/metastasis of tumor cells by blocking β-adrenergic receptors[22]. Propranolol exhibited a greater impact regarding cancer remission when combined with chemotherapeutics and other drugs compared to the single treatments in previous studies conducted with several breast cancer cell lines[23]. In recent studies conducted by Anselmino et al., *in vitro* breast and colorectal cancer models were utilized to test the adjuvant therapeutic effect of propranolol together with either chloroquine or metformin[24,25]. They reported that the combinatory treatment strategy provided a notable reduction in cell viability and migration of cancer cells. In another recent study, propranolol demonstrated a significant anti-tumorigenic effect, only if combined with a chemotherapeutic drug called Irinotecan, on murine colorectal carcinoma cell culture model[26]. Additionally, previous clinical studies have demonstrated that beta adrenergic receptor antagonist propranolol exerted therapeutic effects on breast cancer patients by suppressing proliferative cell marker Ki-67 and also inducing apoptotic pathways[27,28].

Apoptosis is a vital mechanism during embryogenesis as well as a defense mechanism against infections and tumorigenesis by initiating programmed cell death[12]. BCL2 family proteins and caspases are the main mediators of apoptosis. There are also caspase-independent pathways in the cells promoted by mitochondrial nucleases like AIF[16]. It has been known that the disruption of apoptosis via mutations of apoptosis-related players such as p53, or BCL2 results in tumorigenesis and metastasis[12]. In this regard, we checked protein levels of BAX, BCL2, and AIF with ELISA to observe the apoptotic effect of propranolol on MCF-7 tumor cells. The fact that most breast cancers lack CASP3 expression due to the gene deletion makes the apoptosis-inducing agents find alternative pathways like AIF-promoted cell death to multiply the death signal[29]. Therefore, in the current study, we focused on upstream apoptotic players and also a caspase-independent pathway i.e., AIF-induced apoptosis. We showed in the current study that propranolol induced apoptosis by up-regulating pro-apoptotic BAX and AIF proteins and by down-regulating anti-apoptotic BCL2 protein. In line with our study, it has been demonstrated that propranolol significantly triggered apoptotic proteins like BAX in several cell line models while decreasing the anti-apoptotic regulator BCL2[16,30]. Induction of AIF in our study, as an alternative caspase-independent apoptotic molecule, could be upregulated as a backup pathway to support caspase-mediated apoptosis. Furthermore, propranolol blocked the cell survival option of MCF-7 cells by remarkably decreasing BCL2 as the main suppressor protein of the intrinsic apoptotic pathway. In line with the current study, our group has also reported in recent publications that propranolol treatment induced both caspase-dependent and caspase-independent apoptotic pathways via increased caspase-3 and AIF/DDIT3 molecules respectively in human non-small cell lung

adenocarcinoma cell line[31]. In the same study, we also suggested the selective cytotoxic action mechanism of propranolol on cancer cells by sparing most of the healthy lung cells by exerting less cytotoxicity. Besides, we also tested the anti-tumorigenic effect of propranolol on lung cancer cell-derived spheroids and we demonstrated that propranolol caused a notable reduction in cancer spheroid formation efficiency via inducing intrinsic apoptosis[32]. Recent evidence from an *in vitro* study also suggested that an herbal methanol extract exerted cytotoxic effect on a triple-negative breast cancer cell line by inducing intrinsic apoptotic pathway[15].

Emerging evidence indicated that multiple alternative pathways lead the tumor cells to apoptosis. In an *in vitro* cell culture model with gastric cancer cell lines, it was shown that cell proliferation was inhibited after propranolol treatment via the NF-κB pathway by inducing apoptosis and reducing tumor invasion-related matrix-remodeling proteins[33]. BCL2 family proteins, such as BLC2 and BAX, are the main regulators of both mitochondrial and endoplasmic reticulum (ER) stress-induced apoptotic pathways which are mediated by GADD153 and GRP78 pro-apoptotic players[16]. Thus, we also checked the protein levels of GADD153 and GRP78 to analyze alternative apoptotic signals after propranolol treatment. GADD153 (CHOP) is a transcription factor that controls the proliferation, differentiation, and apoptosis of the cells. It was shown in a breast cancer cell line model that GADD153 acted as a pro-apoptotic factor under oxidative stress by activating effector caspases and inhibiting BCL2 via ER[34]. GRP78 is a membrane-bound heat-shock protein that is normally localized in the ER membrane. During tumorigenesis, GRP78 was observed to be upregulated and eventually translocated into cell membranes in breast cancer patients[35]. The re-arrangement of cell membrane protein composition is probably required for breast tumor cells to disseminate further tissues and organs which results in metastasis. Surprisingly, a previous study with triple-negative breast cancer cell lines indicated that GRP78 was also upregulated after treatment with cancer therapeutics like doxorubicin to inhibit tumor proliferation and to trigger apoptotic pathways corroborated by GADD153/CHOP upregulation[36]. In the same study, the blocking of GRP78 by specific antibodies resulted in reduced apoptotic levels in breast cancer cells. Our data showed that protein levels of GADD153 and GRP78 significantly elevated after propranolol treatment in MCF-7 cells. The aforementioned previous *in vitro* studies, along with our data, make us anticipate that GRP78 rearrangement can protect tumor cells by initiating "unfolded-protein response" (UFR) against several stress factors and also metastasis of the primary breast tumor cells. However, GRP78 can also mark the tumor cells as available targets for the apoptotic triggers such as immune cells or cancer therapeutics which might, in this regard, be propranolol in our study.

A previous *in vitro* study claimed that propranolol exerts its anti-proliferative and apoptotic actions by activating cell cycle arrest at G0/G1 and G1/S, checkpoints[37]. To analyze cell-cycle control and proliferation, we measured the protein level of WEE1 in MCF-7 cells. We found that, propranolol treatment significantly increased WEE1 protein levels in breast cancer cells. WEE1 is a kinase protein arresting the dividing cells at the G2/M phase checkpoint if damaged DNA or unfavorable conditions for cell division are present[38]. Previous studies showed that inhibition of WEE1 expression in several breast cancer cell lines drove the cells at the S-phase or G2-phase directly into the M phase without checkpoint regulations. These cells with damaged DNA were forced to apoptosis due to the errors caused by premature mitosis[39]. In this perspective, it was asserted previously that WEE1 inhibitors can be used as promising agents combined with other chemotherapeutics in cancer therapy[39]. On the other hand, it is known that constitutive cell-cycle arrest at checkpoints can also initiate apoptosis[40]. In this context, it can be anticipated that arrest of cell-cycle progression, by e.g., over-expression of WEE1, may lead to a longstanding growth inhibition of cancer cells with an inevitable apoptosis. Overall, elevated levels of GADD153, WEE1, and GRP78 were in line with the augmented levels of apoptotic markers AIF and BAX and reduced BCL2 indicating that tumor growth was interrupted by cell cycle checkpoints long enough to trigger apoptosis.

## Conclusion

In the above study, we investigated the *in vitro* apoptotic and antiproliferative effects of propranolol on human MCF-7 breast cancer cells to analyze its promising antitumorigenic activity. Propranolol exerted its antiproliferative and apoptotic effects on

MCF-7 human breast cancer cells by increasing protein levels of pro-apoptotic factors AIF, BAX, GADD153, and GRP78 and by inhibiting antiapoptotic BCL2. Propranolol treatment also increased WEE1 protein levels which might arrest cell cycle until apoptosis of the tumor cells. Hence, propranolol can be a promising therapeutic agent against breast cancer probably as an adjuvant therapeutic option together with conventional cancer therapy strategies.

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## Conflict of interest

Authors declare no competing interests.

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