Original Article

Pentoxifylline Increased Apoptotic Effects of Tamoxifen on Human Breast Cancer Cells *In vitro*

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Abstract

Aims: Several anticancer drugs are being currently used in the treatment of breast cancer; however, there is a need to develop newer therapeutic regimens that owing to higher cytotoxicity and lower resistance. This study was aimed to investigate the effect of pentoxifylline (PTX) in combination tamoxifen (TAM) on viability and apoptosis of breast cancer cell lines *in vitro*. **Methods:** Two human breast cancer cell lines (MCF-7 and MDA-MB-231) were treated with TAM (2 μM) and different doses of PTX (2, 4, 8, or 16 mM) in combination or alone for 24 or 48 h. Cell viability was evaluated by MTT assay, and effect of these drugs on apoptosis was tested by acridine orange/ethidium bromide staining. **Results:** In 48 h treatment, cell viability of both MCF-7 and MDA-MB-231 cells was reduced in all treated groups ($P < 0.05$). In both cell lines, apoptotic index was significantly increased in combined treatment of 2 μM TAM and 16 mM PTX in comparison with each drug alone ($P < 0.001$). **Conclusion:** PTX induced synergistic effect with TAM on cell growth inhibition of breast cancer cells and could be a candidate drug for breast cancer treatment.

Keywords: *Apoptosis, breast cancer, pentoxifylline, tamoxifen*

Introduction

Breast cancer, the most prevalent malignancy occurring in 14% of women over 30 years old, is induced by the imbalance in cell division and a genetically controlled type of cell death named apoptosis.[1] However, therapeutic clinical techniques involving chemotherapy, radiotherapy, and immunotherapy that have been developed to treat the disease by decreasing the rate of cell division and increasing the number of apoptotic cells could not resolve it up to now. Treated patients showed disease recurrence due to breast cancer stem cell.[2]

Appearances of side effects in normal tissues and drug resistance are the main unsolved problems during chemotherapy for breast cancer patient. In drug resistance condition, the tumors not only do not respond to chemotherapy but also the treatment leads to increase metastasis of nonmetastatic tumors by itself; hence, after a short period of time from beginning of chemotherapy, nonmetastatic tumors become metastatic.[3,4]

Tamoxifen (TAM) has been used as the first-line drug in the treatment of hormone-dependent breast cancers which presents both agonist and antagonist estrogen receptor properties.[4] However, an enduring challenge in the breast cancer treatment field is to identify mechanisms underlying TAM resistance.[5]

Pentoxifylline or 1-(5-oxohexyl)-3,7 dimethylxanthine (PTX), a three-subunit dimethylxanthine derivate, is applied since 1972 for the treatment of vascular diseases by improving arterial outflow; other therapeutic characteristics of the drug are reducing angiogenesis, inflammation, and fibrosis that present PTX for the treatment of various inflammatory cytokines-involved diseases such as diabetes.[6,7]

The anti-inflammatory effects of PTX are due to inhibition of pro-inflammatory cytokines (interleukin 1-beta, interleukin-6, and other mediators) secretion and reduction in superoxide anion production by increasing intracellular levels of cyclic adenosine monophosphate and also downregulating a specific inflammatory intracellular cascade; Toll-like receptor pathway.[8,9] The drug is a vasodilator but its main activity is to reduce the viscosity of blood which probably occurs through influencing on increasing the flexibility of red blood cells, reducing platelet adhesion,

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and improving blood flow and increasing lytic fibrous action in plasma.[10-13]

PTX is also involved in both upregulation and downregulation of apoptosis. The impact of PTX in combination therapy with MS-275 drug on cancer cell lines (HCT-116, MCF-7, PC3, and MDA-MB-231) has been proved by increasing the quantity of the apoptotic cells. The antiapoptotic effects of PTX have been depicted by widely researches on rats showing in liver cells of ecstasy treated, renal cells of methotrexate administrated, myocardial cells of infarcted heart, brain, and intestinal cells of ischemic-induced animals.[14,15] Moreover, the side effects of anticancer drug such as adrenomycin could be attenuated by PTX showing by relieving in cardiomyopathy-induced rats.[16]

These properties of PTX as an anti-inflammatory agent introduce the chemotherapy potential of the drug to increase the efficacy of the anticancer drugs in one side and to overcome the side effects of them in other side. Thus, the present study was conducted to study the *in vitro* effect of PTX on TAM-treated breast cancer cells.

Methods

This *in vitro* study was undertaken in Fertility and Infertility Research Center, Kermanshah University of Medical Sciences. TAM (Sigma ALX-550-095-G001) and PTX (Sigma; 274666-1G) were dissolved in dimethyl sulfoxide at final concentration of 0.1%.

Cell viability assay

Estrogen-positive MCF-7 and estrogen-negative MDA-MB-231 human breast cancer cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2. The identical component of their cell culture media was RPMI1640 enriched with 10% fetal calf serum and penicillin/streptomycin. Consequently, 10^4 cells were grown into each chamber of 96-well culture plates, and following incubation for 24 h, the drug administration was implemented on them in a triplicate manner.

In experimental groups, we also added TAM (2 μM) and different doses of PTX (2, 4, 8, or 16 mM) into the 96-well plates of the cells, and after 24 and 48 h, the cell viability was measured by MTT assay (Sigma; M2003) in respect to the time and the cell types. Briefly, adding 20 μl of MTT solution (5 mg/ml in phosphate-buffered saline [PBS]) to each well, the cells were incubated for 4 h at 37°C. Then, 100 μl of dimethyl sulfoxide was added to each MTT-containing chamber, dissolving formazan crystal at room temperature for 30 min. The optical density of each well was measured by ELISA plate reader at 570 nm. The concentration of TAM (2 μM) was selected according to our previous data that obtained by cell viability measurement (by MTT) and lactate dehydrogenase assay.[17]

Cell morphological analysis

MCF-7 and MDA-MB-231 cell lines were treated with TAM (2 μM), PTX (16 mM), and combination of them either during 24 or 48 h in 96-well plates in a triplicate manner. Then, the wells were prepared for double fluorescent staining with acridine orange (AO)–ethidium bromide (EB). AO is taken up by both viable and dead cells inducing green fluorescence when bound to double-strand DNA in living cells and red fluorescence to single-chain DNA in dead cells. For EB, the dye excludes from living and early apoptotic cells leading to them be stained only with AO. Thus, live cells will show a normal green-stained nucleus while, early apoptotic cells, which have condensed fragmented chromatin, a bright green appearance of compressed disaggregated one. On the other hand, late apoptotic and necrotic cells that their membranes are ruptured allow entering EB dye to stain their nuclei in orange. Subsequently, both emerging dyes intercalate into intact chromatin of late apoptotic and spared DNA of necrotic cells, interacting with each other, to stain the nuclei in sharp or pale red emersions, respectively.[18]

The cells were detached with 0.25% trypsin–EDTA and washed once with PBS. Ten microliters of the cells were then put on a glass slide and mixed with 10 μl of AO (50 mg/ml) and EB (50 mg/ml) monitoring under a fluorescence microscope (Leica, Germany) with ×200 magnification. The percentage of apoptotic and necrotic cells per total number of cells was counted under a fluorescence microscope in five random fields, and mean numbers were considered as apoptotic and necrotic indices, separately.

Statistical analysis

The one-way analysis of the variance using Statistical Software Package, Version 19.0, (SPSS Inc., Chicago, IL, USA) was performed to statistical analyses of the data, and $P < 0.05$ was considered as significance.

Results

Cell viability

The cell viability data of TAM and PTX alone or in combination on both MCF-7 and MDA-MB-231 cell lines achieved by MTT assay are presented in Figure 1. The manner of the effect of the drugs on MCF-7 and MDA-MB-231 cell lines was similar, and there were no statistic differences at same doses and exposure time.

In MCF-7 cell line following 24 h treatment, cell viability significantly reduced in all experimental groups except for 2 mM dose of PTX, where cell viability in PTX (2 mM) + TAM ($P < 0.05$) and PTX 4 mM + TAM, PTX 8 mM + TAM, PTX 16 mM + TAM was significantly declined in comparison with TAM ($P < 0.001$) [Figure 1a]. The cell viability in 48 h treatment of MCF-7 cells was reduced in all experimental groups ($P < 0.001$) besides 2

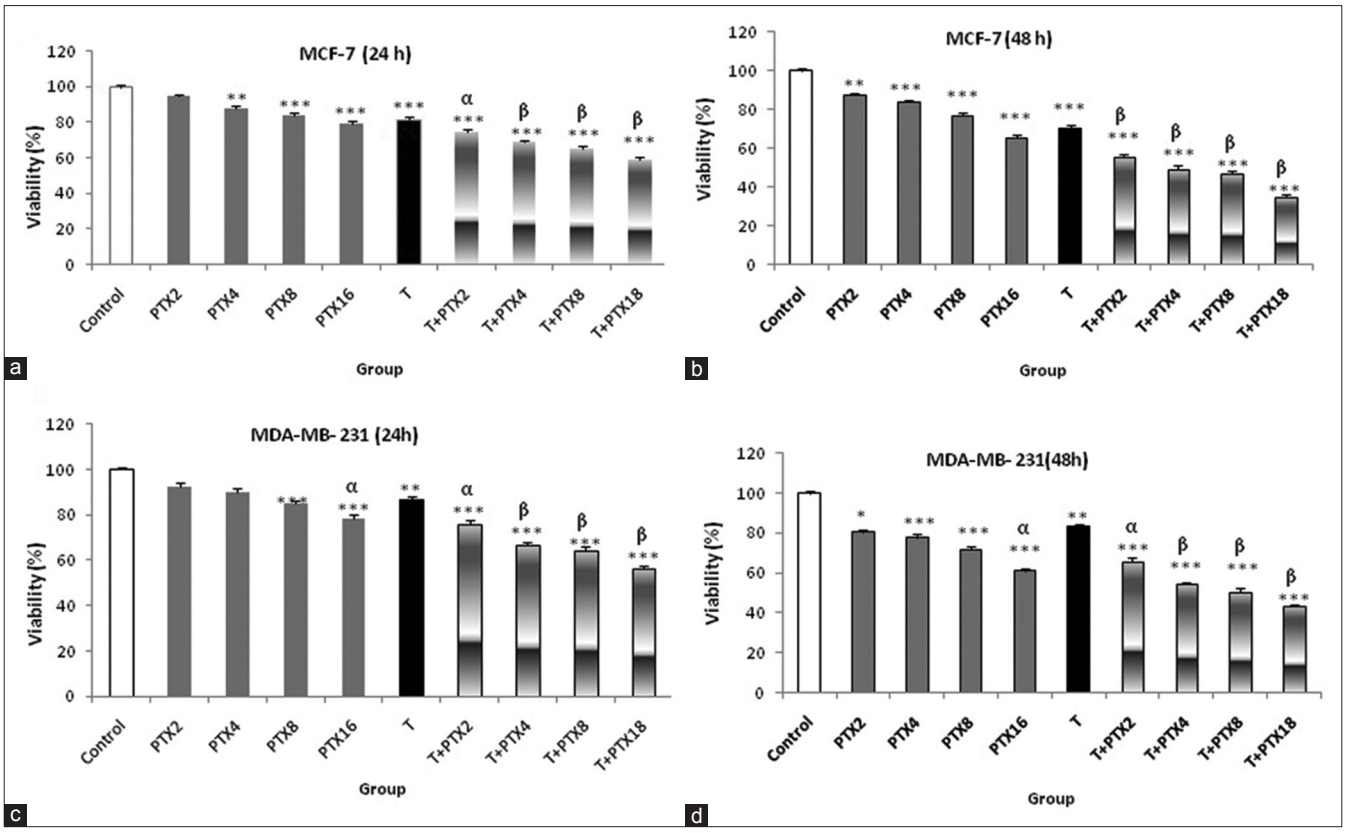

Figure 1: The effects of tamoxifen (2 μM) and pentoxifylline alone or in combination on viability of MCF-7 and MDA-MB-231 cells. Cells were treated with tamoxifen, pentoxifylline, and combination of both for 24 h (a and b) and 48 h (c and d). Control wells were treated with equivalent amount of media alone. Treatment with tamoxifen and pentoxifylline combined significantly decreased the viability compared with tamoxifen or pentoxifylline alone. The results are shown as the mean and standard error of mean from triplicate experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to control and α: $P < 0.05$ compared to tamoxifen, β: $P < 0.001$ compared with tamoxifen

mM dose of PTX ($P < 0.01$) [Figure 1b]. Moreover, all synergic groups showed significant cell viability decline compared with TAM group ($P < 0.001$) [Figure 1b].

In MDA-MB-231 cells, the cell viability after 24 h treatment showed a significant decrease in TAM ($P < 0.01$) group, PTX (8 mM, 16 mM) groups ($P < 0.001$), and all synergistic ones ($P < 0.001$) than control one while, compared to TAM group, cell viability was significantly decreased in PTX 16 mM alone and all synergistic groups ($P < 0.001$) [Figure 1c]. The cell viability of MDA-MB-231 in 48 h treatment was significantly reduced in all experimental groups compared to control group ($P < 0.001$) [Figure 1d]. PTX 16 mM group ($P < 0.01$) and PTX 2 mM + TAM and all other synergic groups also showed significant reduction in cell viability than TAM group ($P < 0.001$). According to these data, the dose of PTX 16 mM was selected for further experiment that was both nontoxic and reduced more cell viability in 48 h.

Detection of cell death in MCF-7 and MDA-MB-231 breast cancer cell lines

The apoptosis stages were detected in MCF-7 and MDA-MB-231 cells treated with TAM and PTX alone or in combination by AO-EB staining [Figures 2 and 3]. The morphological characteristics of cell death in MCF-7 cells were both early and late apoptotic cells in TAM and 16 mM of PTX groups, while in almost all, synergistic group was early apoptosis stage [Figure 2a-d]. In MDA-MB-231, both early and late apoptosis morphologies were occurred in the TAM and 16 mM of PTX groups [Figure 3a-c], and in combination of TAM and PTX 16 mM, a number of cells were in the early stage while few cells in the late stage of apoptosis [Figure 3d].

According to fluorescent staining of MCF-7 with AO-EB, the apoptotic index was increased in PTX 16 mM significantly ($P < 0.05$), TAM ($P < 0.01$), and PTX 16 mM + TAM groups ($P < 0.001$) in comparison with control one [Figure 2e]. Moreover, apoptotic index in PTX 16 mM + TAM was significantly increased in comparison with TAM ($P < 0.05$) and PTX ($P < 0.01$) that showed synergic effect of PTX on apoptotic induction of TAM in MCF-7.

Apoptotic index significantly increased in MDA-MB-231 cells in PTX16 mM ($P < 0.05$), TAM ($P < 0.01$), and PTX 16 mM + TAM groups ($P < 0.001$) in comparison with control [Figure 3e]. Further, apoptotic index was significantly increased in PTX 16 mM + TAM in comparison with TAM and

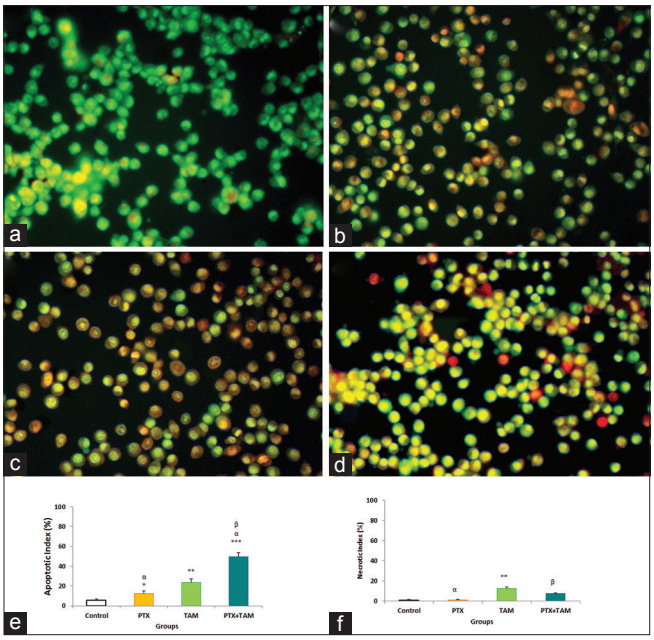

Figure 2: The effects of tamoxifen and pentoxifylline alone or in combination on morphology of MCF-7. The cells were stained by acridine orange/ethidium bromide and observed under fluorescence microscope: (a) Control group; (b) in the presence of tamoxifen (2 μM); (c) in the presence of pentoxifylline (16 mM); (d) in the presence of in combination both. (e) The apoptotic index, (f) the necrotic index based on acridine orange/ethidium bromide stained cells. Arrows show the type of the cells; blue: Live, green: Early apoptotic, yellow: Late apoptotic, and red: Necrotic cells (×200). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to control, α: $P < 0.05$ compared to tamoxifen and β: $P < 0.01$ compared to pentoxifylline group

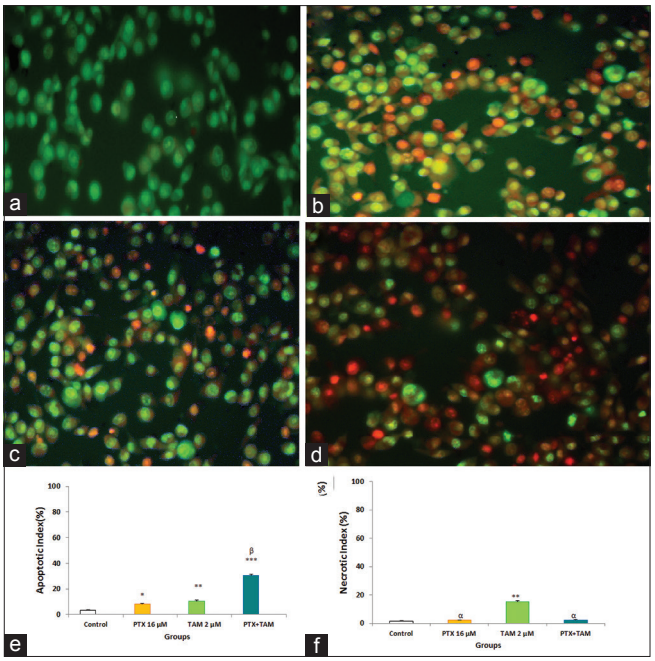

Figure 3: The effects of tamoxifen and pentoxifylline alone or in combination on morphology of MDA-MB-231. The cells were stained by acridine orange/ethidium bromide and observed under fluorescence microscope: (a) Control group; (b) in the presence of tamoxifen (2 μM); (c) in the presence of pentoxifylline (16 mM); (d) in the presence of in combination both. (e) The apoptotic index, (f) the necrotic index based on acridine orange/ethidium bromide stained cells. Arrows show the type of the cells; blue: Live, green: Early apoptotic, yellow: Late apoptotic, and red: Necrotic cells (×200). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to control, β: $P < 0.001$ compared to tamoxifen and pentoxifylline groups, and α: $P < 0.05$ compared to tamoxifen group

PTX ($P < 0.001$) that showed synergic effect of PTX on apoptotic induction of TAM in MDA-MB-231.

The necrotic index was also evaluated in MCF-7 [Figure 2f] and MDA-MB-231 cells [Figure 3f]. In compared to control group, the necrotic index was significantly increased in TAM group ($P < 0.01$) while there were no changes in PTX and TAM + PTX groups ($P > 0.05$) in both cell lines. Moreover, the necrotic index was significantly lowered in PTX and PTX + TAM groups in comparison with TAM group ($P < 0.05$) in both cell lines.

In MDA-MB-231, both early and late apoptosis morphologies were occurred in the TAM and 16 mM of PTX groups [Figure 2b and c], and in combination of 2 μM TAM and PTX 16 mM, few cells were in the early stage while a number of cells in the late stage of apoptosis [Figure 2b and d].

According to fluorescent staining of MCF-7 with AO/EB, the apoptotic index was meaningfully increased in PTX 16 mM ($P < 0.05$), TAM ($P < 0.01$), and PTX 16 mM + TAM groups ($P < 0.001$) compared with control one [Figure 2a and e]. Moreover, apoptotic index in PTX 16 mM + TAM was significantly increased in comparison with TAM ($P < 0.05$) and PTX ($P < 0.01$) that showed an additive apoptotic effect of PTX on TAM in MCF-7 cells [Figure 2a and e].

Apoptotic index significantly improved in MDA-MB-231 cells in PTX 16 mM ($P < 0.05$), TAM ($P < 0.01$), and also PTX 16 mM + TAM groups ($P < 0.001$) in comparison with control [Figure 2b and e]. Further, apoptotic index was significantly increased in PTX 16 mM + TAM in comparison with TAM and PTX each alone ($P < 0.001$) that showed PTX has synergistic effect on TAM for induction of apoptosis in MDA-MB-231 cells.

The necrotic index was also evaluated in MCF-7 [Figure 2a and f] and MDA-MB-231 cells [Figure 2b and f]. In compared to control group, the necrotic index was increased in TAM group ($P < 0.01$) while there were no changes in PTX and TAM + PTX groups ($P > 0.05$) in both cell lines. Moreover, the necrotic index was significantly lowered in PTX 16 mM + TAM group in comparison with TAM group ($P < 0.05$) in both cell lines.

Discussion

The present study indicated that the combination of TAM and PTX has synergistic inhibitory effect on MCF-7 and MDA-MB-231 cells' proliferation by inducing apoptosis. TAM is widely used nonsteroidal anti-inflammatory drugs in the chemotherapy of breast cancer.[19] It inhibits the proliferation of cells by inducing cell cycle arrest in

the G0/G1 phase connected with increased apoptosis. The apoptotic signaling involved downregulation of antiapoptotic Bcl-2, upregulation of Bax, and the activation of caspase-9, -6 and -7, whereas the cell cycle arrest was associated with upregulation of p53 and p21 concomitant with downregulation of cyclin D1 and c-Myc expression.[20]

One of the approaches in this regard is the usage of combination therapies whereby multiple drugs are being delivered at relatively lesser dose that surely confines the aforesaid problems. Studies from tumor models indicate that PTX has a positive effect on cancer treatment. PTX inhibited cell motility of B16F10 melanoma cells by inhibiting F10 invasion, matrix metalloproteinase secretion, and adhesion to matrix components. PTX was found to inhibit migration by inducing protein kinase A activity also affecting RhoA and Rac1 GTPases; this was accompanied with inhibition in RhoA and Rac1 membrane localization.[21]

PTX has antimetastatic and antiadhesive efficacy against breast cancer MDA-MB-231 cells. It affected adhesion of breast cancer cells to extracellular matrix components such as collagen Type IV, fibronectin, and laminin in a dose-dependent manner. The experimental metastasis model using nonobese diabetic-scid mice showed lesser tumor island formation when treated with PTX compared to the control.[22] PTX exhibited antimetastatic activity by affecting processes such as proliferation, adhesion, migration, invasion, and apoptosis. PTX also delays tumor growth and inhibited blood vessel formation *in vivo*.[23] PTX inhibited hypoxia-induced angiogenic factor vascular endothelial growth factor release from A375 melanoma, MCF-7 breast carcinoma, and A549 lung carcinoma cells.[24]

PTX has been shown to increase the effectiveness of antitumor chemotherapy. In a research by Goel and Gude, PTX and liposomal doxorubicin (Lipodox) exhibited synergistic activity and inhibited cellular proliferation to a greater extent with regard to each drug used alone against breast cancer cells *in vitro* and *in vivo*.[25] Combination of MS-275, a histone deacetylase inhibitor, with PTX showed enhanced antiproliferative activity in cancer cell lines colorectal cancer cells HCT-116, prostate cancer PC3, human breast MCF-7, and MDA-MB-231. Further, combination showed enhanced antiangiogenic activity in human breast cancer xenograft model. A significant inhibition of tumor growth was observed in mice bearing MDA-MB-231 breast cancer xenograft treated with the combination of MS-275 (5 mg/kg p.o.) and PTX (60 mg/kg i.p.) than treatments alone, without much signs of toxicity.[26]

PTX has protective effects toward doxorubicin on bacteria and human keratinocytes with no such effects observed on the cancer cells. This reduction in side effects connected without the loss of doxorubicin therapeutic potential.[27] In cervix cancer HeLa and SiHa cell lines, PTX sensitized these cells to chemotherapeutic drug cisplatin-induced apoptosis. Cisplatin induces apoptosis in HeLa and SiHa cells and its effect was significantly increased when the cells were treated with both drugs. PTX diminished expression of antiapoptotic proteins such Bcl-XL and the activation of proapoptotic caspase-3, -6, -7, -9, and -8.[28]

In our study carried out, this combination treatment exhibited synergistic activity and inhibited cellular proliferation to a greater extent with regard to each drug used alone. These results suggest that PTX may be beneficial in breast cancer and suggest that combination of TAM and PTX may target the similar events in breast cancer cells. However, further studies are also needed to investigate molecular mechanisms of PTX in combination with TAM.

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Conflicts of interest

There are no conflicts of interest.

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