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RESEARCH ARTICLE

Pomegranate sensitizes Tamoxifen action in ER-α positive breast cancer cells

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Abstract It is estimated that one in eight women will be affected with cancer during their lives, which means over 1 million women worldwide will be diagnosed with breast cancer in the year of 2011. Roughly, 70% of breast cancer will be estrogen receptor-alpha (ER-α) positive. The presence of ER-α is associated with better prognosis and is able to determine if tumors will respond to the estrogen-blocking/ER-antagonist drug Tamoxifen (TAM). However, a significant fraction of ER-positive tumors respond with minimal or no response to TAM. It is unclear why some breast cancer cells resist TAM and how to make these cells respond. Early evidence suggests Pomegranate fruit extracts (PFEs) exhibit an anticancer effect against some cancers. The objective of the study was to determine whether PFEs may able to enhance/sensitize the TAM's effect in ER-positive MCF-7 breast cancer cells. To test the hypothesis, we determined the effect of PFEs on sensitive and TAM-resistant-MCF-7 cell viability and cell death in the presence or absence of TAM under estrogenic or non-estrogenic culture environment. The present studies demonstrated that PFEs enhance the TAM action in both sensitive and TAM-resistant MCF-7 cells through the inhibition of cell viability (regular or estrogen-induced) by inducing cell-death machinery. Collectively, the results showed for the first time that pomegranate combined with TAM may represent a novel and a powerful approach to enhance and sensitize TAM action.

Keywords Pomegranate · Tamoxifen · Estrogen receptor and breast cancer

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Introduction

Breast cancer (BC) is the most common malignancy among women and second leading cause of cancer related death in American women, after lung cancer (Crowe and Lampejo 1996; Bartow 1999). According to the National Cancer Institute statistics, BC attacks one in eight women, affecting nearly every family worldwide. Approximately 60% of these women will develop the advanced form of the disease, which is fatal (Tavassoli 1999). Despite significant improvement in therapies, many patients experience drug resistance, resulting in disease recurrence, and each year more than 40,000 women will die from this disease [American Cancer Society: *Cancer Facts and Figures 2008*. Available at http://www.cancer.org/docroot/STT/stt_0.asp. 2008]. Accordingly, development of new drugs or enhancements of the efficacy of the existing drugs are the first priority of breast cancer research and management.

BCs are often histologically similar tumors but clinically diverse diseases, and therefore, they may have a different scenario and may respond to therapy differently. It is believed that these disparities are due

to molecular differences between tumors. Accordingly, BCs are classified into four major subtypes based solely on differences in gene expression patterns (Rouzier et al. 2005; Pechoux et al. 1999; Sotiriou et al. 2003). These include Luminal epithelial-like, normal-like, Her2/neu positive and Basal epithelial-like breast cancers (Triple negative). Luminal-like breast cancers are mostly estrogen receptor (ER-α) positive and Her2/neu oncoprotein negative. These cancer cells can proliferate under the influence of estrogen that is obtained from our body directly from internal body sources or outside sources such contraceptive pills, hormone-replacement therapy (HRT), high fat diets or alcohol intake (Hilakivi-Clarke et al. 2002). It is estimated that in 2009 over 1 million women worldwide will be diagnosed with breast cancer, of which about 85% will be classified as luminal-type. Rest of them will be either Her2/neu positive or basal-type breast cancer (Data collected from National Breast Cancer Awareness month issue of ONCOLOGY, 2008). BCs with luminal-type occasionally require a multimodality approach to treatment. These patients are generally treated with hormone therapy including Tamoxifen (TAM). Studies have shown that treatment with approximately 5 years of tamoxifen reduces the annual breast cancer death rate by 31%, irrespective of the use of chemotherapy and/or age (<50 years, 50 to 69 years, ≥70 years), progesterone receptor status, or other tumor characteristics. Unfortunately, adverse effect of TAM as well the resistance to TAM is a huge problem in the treatment of breast cancer patients. Therefore, development of new and innovative remedies to enhance the efficacy of TAM as well as nullify the estrogenic adverse effects is critical to prevent hormone-dependent breast cancer.

The concept of use of alternative therapies is a rapidly growing area in cancer research that seeks new therapeutic approaches to prevent and/or reduce cancer burden (Mehta et al. 2010). There are several herb/fruit medicines that are being tested in various cancer cells under tissue culture condition and animal models to develop new regimen. Pomegranate is one of them (Toi et al. 2003; Khan et al. 2007; Bell and Hawthorne 2008). Pomegranate (*Punica granatum*) fruit extracts (PFEs) exhibit a chemopreventive and chemotherapeutic effects, and these effects are possibly exerted through its antiproliferative, apoptotic, anti-angiogenic or anti-inflammatory action. PFEs have been shown to inhibit the cancerous growth in skin, prostate and breast. Pomegranate seeds are rich in Fatty acid and contain isoflavone genistein and coumestrol, a phytoestrogen. It is also one of the only plants in nature known to contain the sex steroid estrone (Pantuck et al. 2006a, b). Given the importance of PFEs, we can speculate that the combination treatment of pomegranate and tamoxifen may enhance the growth inhibitory effect of tamoxifen in both sensitive and tamoxifen-resistant breast tumor cells. In the current studies, we investigated the effect of PFEs on regular and TAM-resistant-MCF-7 cell viability/growth and apoptosis in the presence or absence of TAM under estrogenic or non-estrogenic culture environment. We find that PFEs enhance the TAM action in both regular and TAM-resistant MCF-7 cells through the inhibition of cell viability (regular or estrogen-induced) by inducing cell-death machinery.

Materials and methods

Reagents Cell culture medium (DMEM; Dulbecco's Minimum Eagles Medium) was obtained from Sigma Chemical Co. (St. Louis, MO) and fetal calf serum (FCS) was purchased from Hyclone Laboratories (Logan, UT). Pomegranate fruit extracts (PFEs) were purchased from Nature's Herbs (American Fork, Utah). Tamoxifen and 17β-estradiol (17β-E2) were purchased from Sigma Chemical Co. All other reagents were purchased from Sigma. Anti-Bax and anti-Bcl-2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Calbiochem (San Diego, CA) respectively. Enhanced chemiluminescence reagents were obtained from Bio-Rad Research Laboratories.

Cell Culture, chemical preparations and treatments Estrogen receptor positive MCF-7 breast tumor cells were purchased from American Type culture collection (ATCC; Manassas, VA, USA) and cultured in DMEM supplemented with 10% FCS and penicillin at 37°C under a humidified tissue culture incubator (Sanyo Inc) containing 5% CO_2. 17β-E2 was dissolved in dimethyl sulphoxide (DMSO) at the concentration of 1 μM/L. Tamoxifen (TAM) was dissolved in DMSO at the concentration of 10 mM/L and Pomegranate extract was dissolved in DMSO at the conc. of 40 mg/ml. Approximately 70% confluent cells were treated with different chemicals with appropriate doses and times as per the requirement of the experiment indicated in the experimental designs.

Cell Proliferation analysis by cell counting using 0.4% Trypan blue (TB) MCF-7 breast tumor cells (10,000 cells per well in 3 ml medium) were plated onto 24-well corning tissue culture plates containing regular media. Cells were grown into a CO_2-incubator over night for attachment. The cells were then grown in phenol red free DMEM containing 5% charcoal-treated stripped FCS until the culture became 70% confluent. The later culture condition creates an estrogen-free environment, which is necessary to avoid estrogenic action of serum or phenol red on cells (Thompson et al. 1989a, b). After reaching ~70% confluent

growth, cells were treated with different drugs including 17β-E2, TAM and PFEs for indicated time points as per the requirement of the experiments. After completion of the experiments, cells were stained with 0.4% trypan blue solution for 30 min and counted the viable cells (unstained) using cell counter (Nexcelom).

Analysis of cell cycle by flow cytometry MCF-7 cells were seeded at a confluency of 70% in 75 mm T-Flask. After overnight incubation, cells were either left untreated or treated with 1 μM TAM, 300 μg/ml PFEs or the combination in the presence or absence of 10 nM 17β-E2. After 48 h, cells were trypsinized, washed with cold 1x PBS (phosphate buffer saline), resuspended in 2 ml of ice cold 1× PBS, fixed by three stepwise additions of 2 ml each of 95% ethanol, and stored at 4°C for cell cycle analysis. Subsequently, cells were resuspended in 1 μg/ml RNase (Sigma) in PBS to destroy RNA in the cells and stained with 0.05 μg/ml propidium iodide (Sigma) for 1 h on ice. Flow cytometric analysis was performed with a Fluorescence-activated cell sorting (FACS) flow cytometer (Becton-Dickinson, San Jose, CA) as described earlier (Ormerod 2004). A minimum of 15,000 cells was analyzed for each sample. Data analysis was performed using Modfit 5.2 analysis software (Verity Software House, Topsham, ME). Each point represents at least triplicate samples from a single experiment.

Annexin V: FITC assay for program cell death Untreated and treated cells were stained with Annexin V, diluted with binding buffer (1:40 dilution) for 10 mins. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for phospholipid-like phosphatidylserine (PS), and binds to cells with exposed PS that found in apoptotic cells. After staining, the cells were washed and incubated with binding buffer containing Propidium iodide solution for another 10 min. Cells were washed, mounted in mounting solution and examined under fluorescence microscope to visualize apoptotic cells. Green color cells are considered early stage of apoptotic cells while red color is considered late. Each point represents at least triplicate samples from a single experiment.

Detection and quantitation of Bcl-2 and Bax proteins by Western blot analysis For western immunoblot analysis, cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS containing PMSF (1 mM), leupeptin (1 μM) and Aprotinin (1 μM)] for 30 mins with occasional vortexing according to the previous method (Sengupta et al. 2004a, b). Protein concentrations in supernatants were measured using the coomassie blue reagent assay as per the instruction of the company (Pierce Chemical). Equal amounts of untreated (control) and treated proteins were run in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membrane and reacted with anti-Bcl-2 and Bax antibodies. Immunoreactions were detected by ECL reagent kit (Pierce, Rockford, IL) and relative expression of proteins was calculated by densitometric analyses using ID image Analysis Software version 3.6 (Eastman Kodak Company, Rochester, NY). Each point represents at least triplicate samples from a single experiment.

Statistical analysis Data were analyzed by Student's *t* test; A probability level of $p<0.05$ was considered significant. Error bars represent standard error of the mean (SEM).

Results

Effect of TAM, PFEs and the combination on cell growth of ER-positive, TAM-sensitive MCF-7 breast cancer cells in regular and estrogenic environment It is well-established that estrogen is a strong mitogen for ER-α-positive MCF-7 breast tumor cells and this mitogenic action of estrogen can be blocked by TAM (Thompson et al. 1989b; Dickson et al. 1989; Hayes and Robertson 2002). In addition, TAM is also capable of blocking the estrogen-independent regular cell growth (constitutive) of ER-positive, TAM sensitive MCF-7 cells (Hayes and Robertson 2002). Therefore, the goal of this study was to determine whether PFEs are able to show a synergistic or additive effect on the growth inhibitory effect of TAM in MCF-7 cells in the presence or absence of estrogen. To test the objective, three experiments were performed. First, ~70% confluent MCF-7 cells, that were grown in an estrogen-free environment, were treated with 17β-E2 (10 nM), TAM (1 μM), PFEs (300 μg/ml) alone or in combinations (i.e., 17β-E2+TAM; 17β-E2+PFEs; 17β-E2+TAM+PFEs and TAM+PFEs) or left untreated (vehicle control) for 48 h. The doses of these three variables were found from previous studies (Banerjee et al. 1997). Cells were then incubated with 0.4% trypan blue (TB) solution for 30 min to stain the dead cells. After staining, we counted unstained cells as an index of viable cells using cell counter (Nexcelom). As shown in Fig. 1, 17β-E2 (10 nM) significantly ($p<0.01$; paired student's *t*-test) enhances the MCF-7 cell proliferation as compared to untreated control cells. Our result is consistent with previous studies (Thompson et al. 1989a). TAM and PFEs significantly reduced the constitutive as well as estrogen-induced MCF-7 proliferation. Combination treatment of TAM and PFES exhibits additive growth inhibition effect

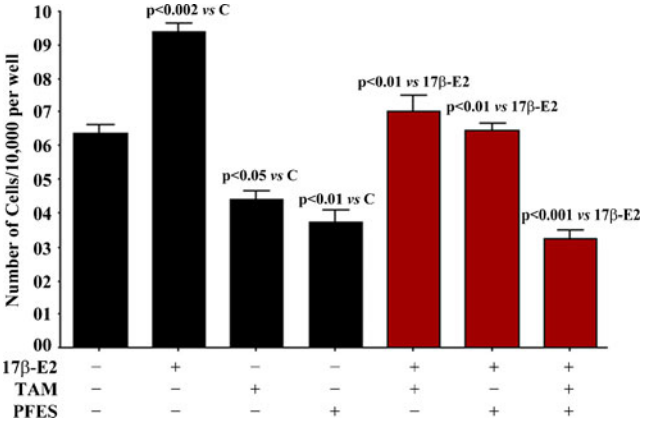

Fig. 1 The additive effect of PFEs on TAM-induced inhibition of mitogenic action of estrogen on MCF-7 cells. The *bar graph* represents the cell proliferation of MCF-7 cells before and after the treatment of 17β-E2 (10 nM), TAM (1 μM) or PFEs (300 μM) alone or in combination for 48 h. Untreated cells were exposed to vehicle controls (DMSO). Data are displayed as means ± SE of at least four independent experiments. *P* values were determined by a Student's *t* test

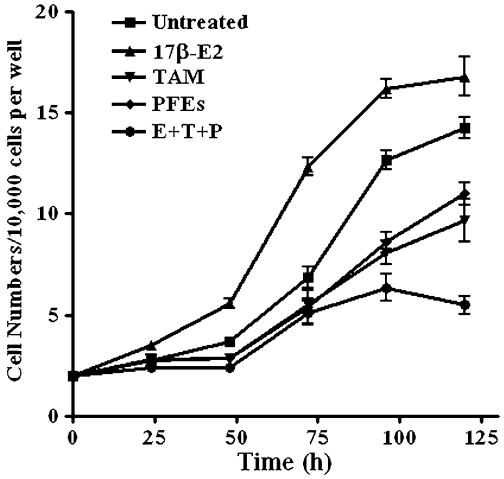

Fig. 2 Time-dependent effect of PFEs on TAM-induced inhibition of mitogenic action of estrogen on MCF-7 cells. MCF-7 cells were treated with or 17β-E2 (10 nM), TAM (1 μM) or PFEs (300 μg/ml) alone or in combination for different times and cell counts were determined using automated cell counter (Nexcelom) as described in the Materials and methods section. Data are shown as the means of three separate experiments; *bars* represent SE. *Significantly different from treatment with 17β-E2 ($p<0.001$ by a Student's *t* test.). #significantly different from treatment with Untreated ($p<0.05$ by a Student's *t* test)

on both constitutive and 17β-E2 -induced proliferation. Second, we determined the time-dependent effect of these chemicals. We found that PFEs significantly enhance the growth inhibitory effect of TAM in a time-dependent manner. The additive effect was first detected at 24 h and increased subsequently as the time progressed (Fig. 2). Finally, we explored the effect of TAM (1 μM) or PFEs (300 μg/ml) or combination of tamoxifen and pomegranate extracts on cell-cycle distribution in 17β–E2-treated and untreated MCF-7 cells using flow cytometry analysis. Tamoxifen is known to cause accumulation of cells in the sub-G0-G1 phase, which is an indication of cell arrest and/or apoptotic cell death (Taylor et al. 1983). Our results showed hat at 48 h (Fig. 3) of TAM or PFEs exposure, both drugs individually resulted in a statistically significant increase in the percentage of cells in sub-G0-G1 compared with untreated or estrogen treated cells ($P<0.01$). The combination of TAM and PFEs produced a statistically significant further increase in the percentage of cells in G0-G1 phase compared with the effect of either drug alone. The cells in G0-G1 decrease significantly in estrogen exposed cells as compared to untreated cells. At 48 h, there was also a decrease in the percentage of cells in S phase compared with untreated cells.

Effect of TAM, PFEs and the combination on apoptosis in 17β-E2-treated or untreated MCF-7 cells Since the percentage of sub-G0-G1 phases increased significantly in individual treatment of TAM and PFEs and additive effect was seen by their combination treatment, we hypothesized that TAM and PFEs treatment might cause apoptosis in MCF-7 cells and this effect could be enhanced by combination treatment. The goal was to confirm the hypothesis by analyzing the apoptosis in these cells. To do so, semi confluent MCF-7 Cells were cultured in chamber slides in the presence of 1 μM TAM, 300 μg/ml PFEs, both chemicals, or vehicle control under regular or estrogenic environment for 48 h. The

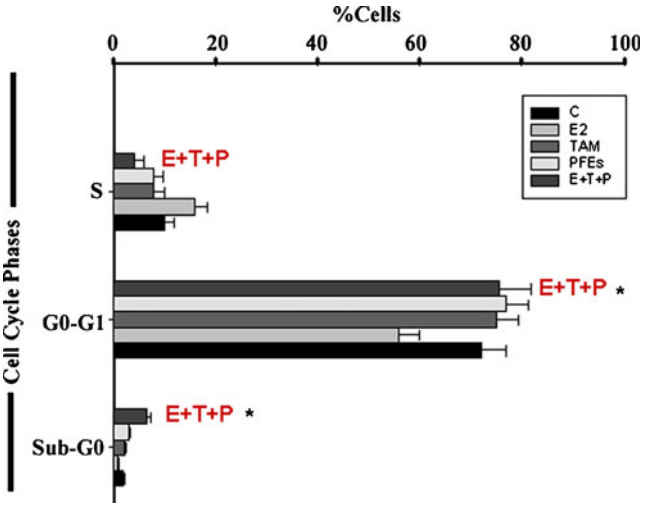

Fig. 3 The additive effect of PFEs on TAM-induced regulation of cell cycle phases in estrogen treated MCF-7 cells. The *bar graph* illustrates the effect of TAM (1 μM) or PFEs (300 μg/ml) or combination of TAM and PFEs on cell-cycle distribution in 17β-E_2-treated (10nM) and untreated MCF-7 cells. The cell cycle phases were analyzed in DAPI-stained MCF-7 cells using flow cytometry. Data are shown as the means of three separate experiments; *bars* represent SE. *Significantly different from treatment with 17β-E2 ($p<0.01$ by a Student's *t* test)

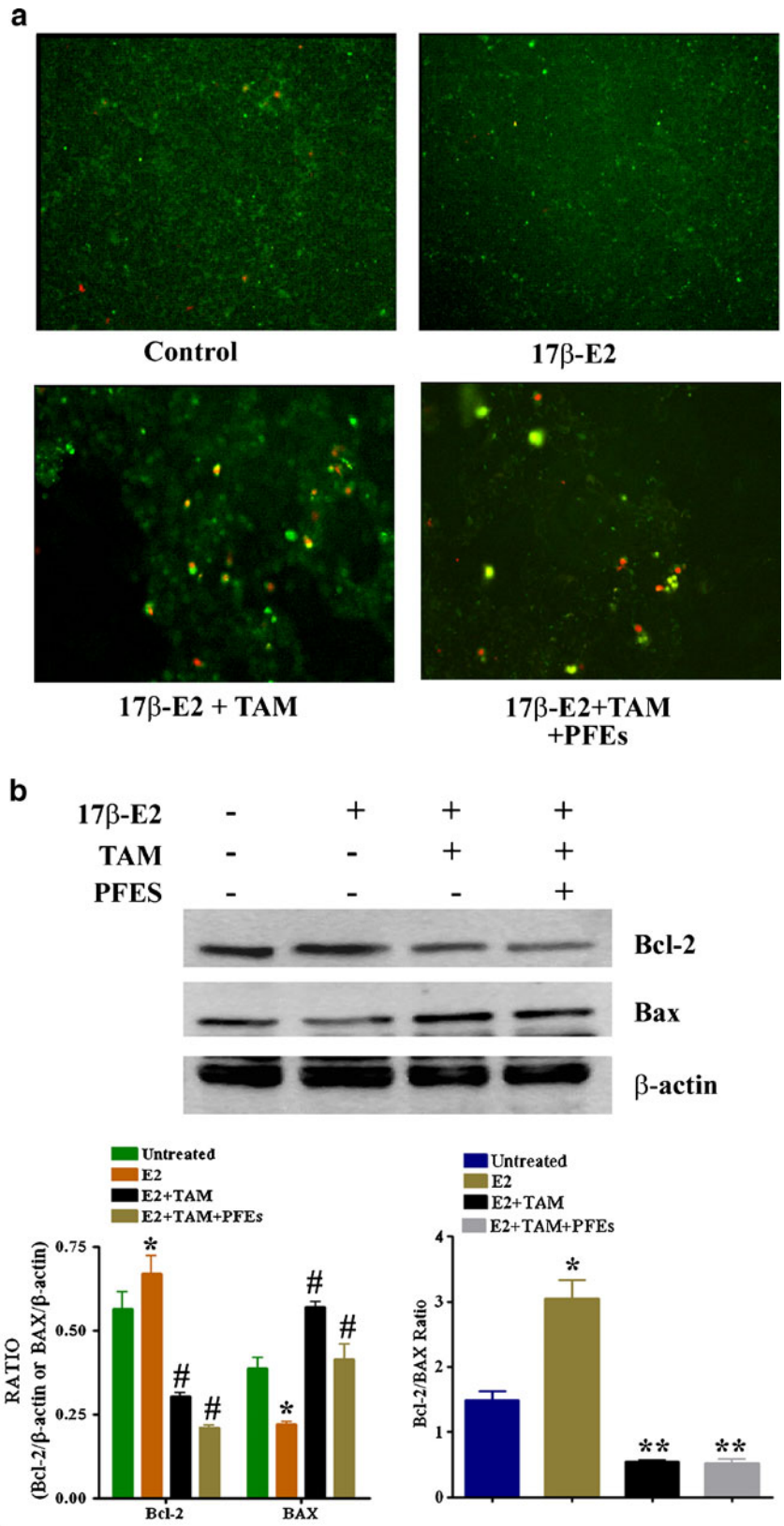

Fig. 4 The additive effect of PFEs on TAM-induced apoptosis in estrogen treated MCF-7 cells. **a**. Annexin V: FITC assay for the detection of early (*green*) and late apoptosis (*red*) in MCF-7 cells. The upper panel (left and right side) represents without or with 17β-E2 treated cells respectively. The lower panel (left and right side) represents the cells treated with 17β-E2 and Tam and 17β-E2, Tam and PFE together, respectively. **b**. Immunoblot analysis represents the expression of Bcl2 and Bax protein. β-actin was used for loading control. The left *bar graph* represents the Bcl-2/β-actin or BAX/β-actin ratio while right *bar graph* exhibits the ration of Bcl-2 and BAX in treated and untreated samples. Data are shown as the means of three separate experiments; *bars* represent SE. *Significantly different from treatment with Untreated ($p<0.05$ by a Student's *t* test.), #significantly different from treatment with 17β-E2 ($p<0.005$ by a Student's *t* test.) and ** significantly different from treatment with 17β-E2 ($p<0.001$ by a Student's *t* test)

cells were fixed in methanol and stained with Annexin V followed by Propidium iodide as counter stained for apoptosis analysis. As shown in Fig. 4, no apoptotic cells were detected in control and estrogen-exposed cells. However, apoptosis was increased significantly in both TAM and PFEs exposed cells and this effect was markedly increased when cells were

treated in combination of TAM and PFES. To confirm this result, additional assays of apoptosis were used. Cells were cultured as above, and protein extracts were analyzed for Bcl-2 and Bax proteins by immunoblotting with anti-Bcl-2 or anti-Bax antibodies. We detected these two proteins by immunoblotting because Bcl-2 is considered as anti-apoptotic protein while Bax is a pro-apoptotic protein. Expression profile ratio of these two proteins is crucial in induction of apoptosis. Our studies showed that 10nM 17β-E2 enhances Bcl-2 protein expression and at the same time blocks Bax protein expression in MCF-7 cells after 48 h of treatment. 1 μM TAM and 300 μg/ml PFEs inhibit 17β-E2 - induced Bcl-2 expression and reduce the inhibitory effect of estrogen on Bax expression in MCF-7 cells. Combination treatment exhibits an additive effect on these two proteins (Fig. 4). The constitutive (non-induced) expression of Bcl-2 and Bax is also affected by TAM and PFEs (data not shown).

Effect of TAM, PFES and combination on the proliferation of TAM-resistant MCF-7 cells The objective of this study was to determine whether PFEs are able to rescue the cells from TAM resistance through the induction of efficacy of TAM. To do so, we used a TAM resistant MCF-7 cell line (MCF-7rTAM). A TAM-resistant MCF-7 variant was established in this laboratory selecting against increasing concentrations of TAM for 3 months (Fig. 5a). After establishing, these cells were grown in media contained 500nM TAM. However, this cell line was cultured in DMEM in the absence of TAM for 1 week before experiment. Cells were then treated with TAM (10 mM), TAM+PFEs (300 μg/ml), or no drug (control) for 72 h and cell viability was determined. The studies showed that MCF-7rTAM cells are no longer responsive to the antiproliferative effects of TAM (Fig. 5c). However, this non-responsive behavior of TAM was changed when cells were treated in combination of PFES and TAM, and almost 50% inhibitory effect was detected in combination therapy. PFEs alone also exhibit inhibitory effect in these cells but this effect was significantly less than combination treatment.

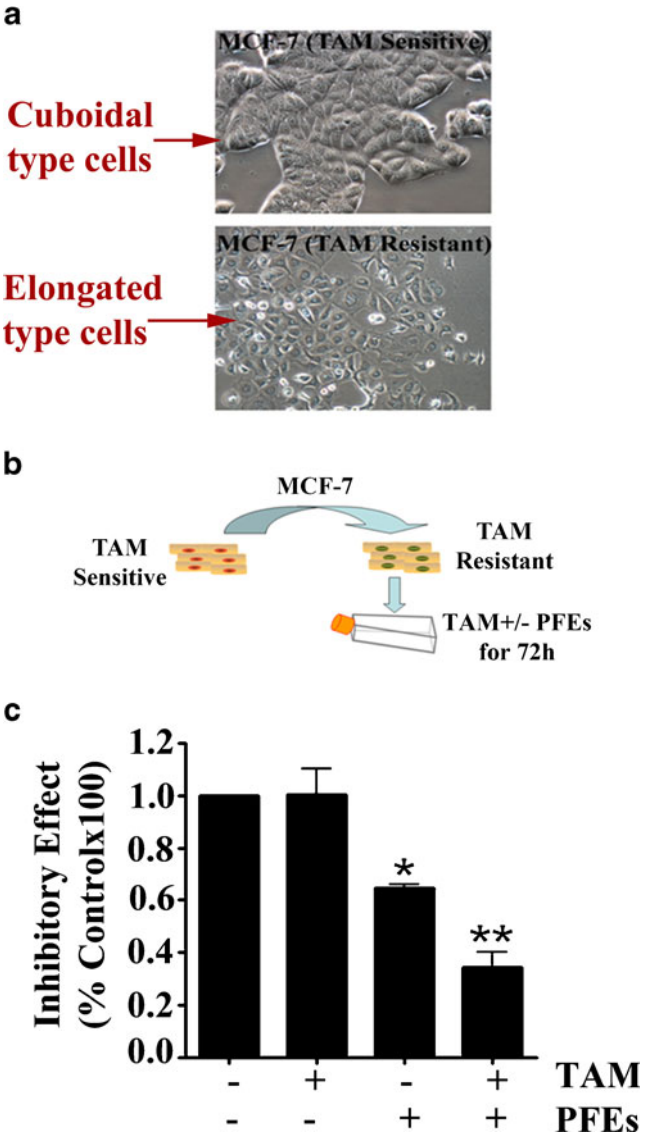

Fig. 5 PFEs sensitize TAM action on TAM-resistance MCF-7 cells. **a**. Photographs are showing the morphological difference between Tamoxifen sensitive and resistant MCF-7 cells. **b**. A diagrammatic representation of the experiments and **c**. The *bar graph* represents the cell proliferation assay in Tamoxifen-resistant MCF-7 cells treated with TAM in the presence or absence of PFEs for 72 h. Data are shown as the means of three separate experiments; *bars* represent SE. *p* values were determined by a Student's *t* test. * indicates a *p* of <0.01 vs untreated or control; ** indicate a *p* value of <0.001 vs PFE

Discussion

The present studies have demonstrated three important effects of PFE on breast cancer cells under the tissue culture condition. These are: (1). PFE is able to nullify the cancer promoting effects of estrogen. These include hyper proliferation and inhibition of program cell death process through the mal-regulation of proliferation and apoptosis related genes in estrogen receptor positive cells, (2). The combination of TAM, a selective estrogen receptor modulator (SERM) and a conventional medicine for estrogen receptor positive breast cancer patients, and PFEs resulted in additive growth inhibition and augmentation of cell accumulation in the sub-Go (apoptosis) and G1(resting phase) phases of cell cycle, with a decrease in S phase, and (3). PFEs are able to enhance the inhibitory effect of TAM in TAM-resistant breast tumor cells.

Tamoxifen (TAM) is a drug that is used to treat women and man with breast cancer and interferes with the activity of estrogen. The main use for the drug is during the early and metastatic stages of breast cancer. For women that are

at risk for breast cancer, TAM is given to the patient to reduce the chances of developing the cancer (Ali and Coombes 2000; Ali et al. 2000). Although the precise mechanism of action of TAM is still unknown, pioneer studies have shown that the positive effect of estradiol on proliferation and Bcl-2 expression on ER-positive breast cancer cells could be blocked by TAM through preventing the interaction of estradiol and its receptor (Wang and Phang 1995). Our in vitro studies, using ER-α-positive-MCF-7 cells as a model, indicate that under estrogen free tissue cultured environment 17β-E2 stimulates MCF-7 cell proliferation and inhibits apoptosis through possibly by modulation of Bcl-2 and Bax ratio and these effects can be significantly blocked by TAM (Figs. 1, 2, 3 and 4). These results are partially consistent with previous studies (Wang and Phang 1995; Zhang et al. 1999) as the previous studies found no effect of TAM on BAX expression, which is, however, documented in our present studies. Furthermore, the studies showed that PFEs mimic the TAM's action and significantly reduced the effect of estradiol on MCF-7 cells. The combination of TAM and PFEs resulted in additive growth inhibition and augmentation of apoptosis.

Although TAM is one of the standard therapeutic options that used for adjuvant therapy of ER-α positive breast cancer patients as well as chemoprevention of high-risk women (Tormey et al. 1996; Nayfield 1995; Hussain et al. 2007), acquired hormone resistance reduces TAM activity through complex mechanism(s) (Kumar et al. 2009), and treatment-associated side effects limit use of high doses TAM (Ali and Coombes 2000). Therefore, more effective modality is needed to sensitize TAM on ER+/TAM-resistant breast tumors. Combination treatment is one of the developmental trends for increasing the efficacy and/or minimizing the side effects. The present studies demonstrate that PFEs are able to enhance the inhibitory effect of TAM in TAM resistance breast tumor cells. Selection of MCF-7 cells against TAM led to the establishment of the MCF-7rTAM cell line, which is stably resistant to TAM. In the resistant MCF-7rTAM cells, significant morphological changes are observed (Fig. 5). TAM-sensitive MCF-7 cells are round epithelial type cells. In contrast, MCF-7rTAM cells are spindle-shaped mesenchymal type cells. The morphological changes suggest that TAM resistant cells may achieve certain aggressive behaviors (Christofori 2006). However, further studies are warranted. We found that when MCF-7rTAM cells were exposed to TAM, minimal or no effect on cellular growth of MCF-7rTAM cells was detected. Interestingly, the TAM can regain its antiproliferative effect in the presence of PFEs (Fig. 5). Although, the mechanism of the action of PFEs in this transition in unknown, studies suggest that PFEs diminish the molecular barriers that are associated with the resistance to antiestrogen. To uncover this mechanism, further studies are needed.

Collectively, these studies suggest that use of pomegranate as an alternative combination therapy may represent a novel and a powerful approach to the treatment of ER positive breast cancer by enhancing TAM actions.

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