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Combinational treatment of gap junctional activator and tamoxifen in breast cancer cells

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Abstract

Introduction—Tamoxifen is a drug of choice for endocrine-responsive breast tumor patients. However, tamoxifen resistance has become a major concern for the treatment of breast cancer. Combinational therapies of tamoxifen and different drugs are being frequently studied. In the current study, we tested the efficacy of substituted quinolines (code name = PQ1; gap junctional activator) in combination with tamoxifen in T47D cells.

Methods—Colony growth assay was performed using soft agar to measure the colony growth while cell proliferation was measured by MTT assay in T47D cells. The level of Ki67, survivin and BAX was measured using confocal microscopy along with western blot analysis. APO-BrdU labeling was also examined in the induced treatment of T47D cells.

Results—We observed a 55% decrease in the colony growth in the presence of combination of PQ1 and tamoxifen; while tamoxifen alone has little effects. Combination of 10 μ M tamoxifen and PQ1 200 nM or 500 nM resulted in only 16% cell viability compared to controls at 48 hr in T47D cells by MTT assay. We found a significant increase in BAX protein at 1 hr in the presence of 500 nM PQ1 alone, 10 μ M tamoxifen alone and combination of PQ1 and tamoxifen. A 2-fold increase was observed in active caspase 3 in the presence of combinational treatment of 10 μ M tamoxifen and 200 or 500 nM PQ1. Also, flow cytometric analysis showed a 50% increase in the number of apoptotic cells in the presence of combination of tamoxifen and PQ1 compared to the control. Furthermore, the results show that combinational treatment of tamoxifen and PQ1 significantly reduces the expression of survivin in T47D cells.

Conclusions—The combinational treatment of PQ1 and tamoxifen has a significant increase in BAX expression, caspase 3 activation and DNA fragmentation. Tamoxifen alone and combination with PQ1 showed a decrease in the survivin expression while PQ1 alone shows to be independent of survivin-mediated pathway. This suggests that an increase in gap junction activity can potentiate the effect of tamoxifen. The combinational treatment of tamoxifen and PQ1 also showed a significant decrease in cell viability compared to tamoxifen treatment alone. The present study demonstrates for the first time that combinational treatment of tamoxifen and PQ1 (gap junctional activator) can be used to potentiate apoptosis of T47D human breast cancer cells. Thus,

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gap junctional activator, PQ1, could alter either the length or dose of tamoxifen clinically used for breast cancer patients.

Keywords

Breast cancer; Tamoxifen; Caspases; Apoptosis; Gap junctional activator; PQ1

Introduction

Tamoxifen is one of the most commonly and successfully used chemotherapeutic agent for the treatment of endocrine responsive breast cancers [1]. Tamoxifen is better known as a selective estrogen receptor modulator (SERM) because of its multiple activities [2]. In most patients, cancers that initially respond to tamoxifen gradually acquire resistance to the treatment and require alternative systemic therapies [3]. Despite extensive use of this drug, the precise mechanisms that confer resistance remain unknown. A number of mechanisms has been proposed to control antiestrogen resistance in estrogen receptor positive (ER⁺) breast cancer [4], but many details of these mechanisms continue to be unclear [5]. These include changes in the host immunity, host endocrine system, or antiestrogen pharmacokinetics [1]. Under host endocrine system, some tumors spontaneously become hormone-independent despite the presence of ER; in others, tumors that are initially ER⁺ become ER⁻ over time [6, 7]. Numerous trials have been conducted using the combinational treatment of chemotherapy plus tamoxifen but the results have been controversial [8-13].

Gap junctions are the intercellular plasma membrane channels which allow the passage of small molecules from one cell to other. The flux of molecules through the channels is called the gap junctional intercellular communication (GJIC) [14]. GJIC exists in most of the mammalian cells and is involved in cell growth, differentiation, and homeostasis. For decades it has been shown that the mitotic cells in the cell cycle show decreased GJIC [15-17]. Therefore, it leads to a state that the cell-cell communication is negatively related to the capability of the cell to grow. Due to its effect on the cell growth, many studies were conducted to find a co-relation between the GJIC and cancer. Many *in vitro* and *in vivo* studies showed that tumor-promoting agents lead to a decrease in GJIC [18-21] [22, 23]. Protein and mRNA analysis showed a decrease in connexin expression in preneoplastic lesions as well as hepatocellular carcinomas [24, 25].

In previous study we reported a new gap junctional activator, substituted quinoline (code name = PQ1). We demonstrated that PQ1 (200 nM) caused a 70% increase in the GJIC in T47D cells; however, there was no effect of PQ1 treatment on GJIC in normal mammary epithelial cells. In addition to an increase in GJIC, 80-95% growth attenuation was observed by PQ1 in colony growth assay. Moreover, an increase in caspase 3 with PQ-treated cells was observed, suggesting a possible involvement in apoptosis as well as increasing gap junction activity [26].

The antitumor effects of tamoxifen are thought to be due to its antiestrogenic activity, mediated by competitive inhibition of estrogen binding to ER and subsequently activation of apoptosis [27] [28]. The inhibition of expression of estrogen-regulated genes causes decrease in cell growth and proliferation [29]. Since PQ1 have also shown an increase in

caspase-3 and a decrease in breast tumor growth, we hypothesize that combinational treatment of tamoxifen and the gap junctional activator, PQ1, increase on T47D human breast cancer cells. The goal of the present study is to examine the effect of combinational treatment of PQ1 and tamoxifen in T47D breast cancer cells. The results showed that an increase in T47D cell death was observed in combinational therapy of tamoxifen and PQ1. A significant increase in BAX and caspase 3 followed by an increased apoptosis by APO-BrdU incorporation at different dosing time was observed in the presence of PQ1 and tamoxifen. Furthermore, a decrease in the colony growth, MTT assay and an increased DNA fragmentation were occurred in the combinational treatment of PQ1 and tamoxifen.

Materials and Methods

Cell Lines and Culture

The T47D human breast cancer cell line was purchased from American Type Cell Culture (ATCC, Manassas, VA). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10% antibiotic-antimycotic at 37°C with 5% CO₂ in 75 cm² flasks. Tamoxifen citrate was purchased from Sigma (Saint Louis, MO).

Cell Morphology

Cells (5000 cells/ml) were seeded in a 6-well plate and dosed with 200 nM PQ1 alone, 10 µM tamoxifen alone, combination of tamoxifen and PQ1, and combination of tamoxifen and estrogen (10 nM) for 24, 48 and 72 hr. Cells were observed under a microscope at 40X objective.

Colony Growth Using Soft Agar Assay

Cells were treated with ethanol, 200 nM PQ1, 10 µM tamoxifen, and combination of 200 nM PQ1 and 10 µM tamoxifen for 7 days. Base agar plates were prepared containing 0.8% agar and 0.4% agar in RPMI. Cells (5×10^4 cells/33 mm² well) were suspended in 100 µl of RPMI with 0.4% agar and plated. These plates were maintained at 37°C for 7 days and examined for the presence of colonies. Individual colonies of 50 µm or greater were examined.

MTT Assay

The MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed with cell cultures in a 96-well plate incubated with 200 and 500 nM PQ1, 10 µM tamoxifen alone, and combination of 10 µM tamoxifen and 200 or 500 nM PQ1 for 1, 48 and 72 hr. The MTT solution was metabolized by the cells (incubation period 1 hr) at 37°C. MTT is a tetrazolium salt (yellowish) cleaved to formazan crystals by succinate dehydrogenase. In viable cells, more formazan dye will be produced. After solubilization of MTT crystals with the 0.35 N HCl solubilization solution, dye was measured spectrophotometrically at 540 nM with the background subtraction at 650 nM.

Western Blot Analysis

Cells were grown in serum-supplemented RPMI media until they were 90% confluent in 25-cm² flasks. Cells were incubated with PQ1 alone, 10 μ M tamoxifen alone, and combination of tamoxifen and PQ1 for 1, 48 and 72 hr. Cells were washed 3 times with cold PBS and harvested using cell lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100) with 1:1,000 dilution of protease inhibitors (Sigma-Aldrich, St. Louis, MO) for 10 min followed by rotation on a shaker for 30 min at 4°C. Cells were vortexed and centrifuged at 13,000 rpm for 30 min at 4°C. Forty μ g of whole cell extract was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, St. Louis, MO). Nitrocellulose membrane was blocked in 5% milk for 1 hr at room temperature and incubated with polyclonal rabbit Bcl2, (1:200), mouse BAX (1:200, Santa Cruz Biotechnologies, Santa Cruz, CA), polyclonal rabbit caspase 3 (1:500, BD Pharmingen, San Diego, CA), polyclonal rabbit survivin (1:1,000, Novus Biologicals, CO, USA) and polyclonal rabbit actin (1:1,000, Sigma-Aldrich, Saint Louis, MO). Western blots were detected by enhanced chemiluminescence (ECL) detection (Amersham, Pittsburg, PA).

Immunofluorescence and Confocal Microscopy

Cells were grown on coverslips in 6-well plates in RPMI media. Cells were treated with PQ1 alone, tamoxifen alone, and combination of tamoxifen and PQ1 for 1, 48 and 72 hr. Cells were fixed with 2% paraformaldehyde for 20 min and then neutralized with 50 mM glycine for 5 min. The cells were lysed with 0.1% Triton X-100 for additional 10 min. After washing with PBS, cells were blocked with 2.5% BSA in PBS for 2 hr and then incubated with primary antibodies, rabbit Ki67 (1:250, Santa Cruz, CA) and mouse BAX (1: 50, Santa Cruz, CA), rabbit survivin (1:250, Novus Biologicals, CO, USA) for 15 hr at 4°C. Following this step, cells were incubated in DAPI for a minute and then incubated with anti-mouse and anti-rabbit Alexa fluor 488 and 568 (Molecular probes, Eugene, OR, USA) for 4 hr at 4°C, respectively. Cells were analyzed for nuclear morphology by staining with DAPI. Samples were sealed and analyzed by a confocal microscope (Carl Zeiss LSM 510 META, Narashige, MN).

Apoptosis Assay by Flow Cytometry

Cells were grown in a 35 mm² dish and then dosed with PQ1 alone, tamoxifen alone, and combination of tamoxifen and PQ1 for 1, 48 and 72 hr. Cells were trypsinized and stained with APO-BrdU TUNEL Assay kit (Molecular Probes # A23210, Carlsbad, CA) according to the manufacturer's protocol. APO-BrdU binding was analyzed by flow cytometry using a BD FACSCalibur system and the data obtained was analyzed using the CellQuest software.

Results and Discussion

Breast cancer is the second leading cause of cancer deaths in North American women. Tamoxifen has been the single agent of choice in treating hormone responsive breast cancer cases since 1971. Even though tamoxifen has been shown to be 50% more effective than placebo in preventing the occurrence of breast cancer in high-risk population, the risk of developing uterine cancer has increased by more than 40% [30]. The line of treatment with

tamoxifen includes the usage of the drug for at least 5 years in most of the patients. Long-term treatment with tamoxifen induces tamoxifen resistance, the mechanism of which is still being elucidated [2]. Therefore, it is necessary to develop effective modalities to enhance the efficacy of tamoxifen. In our present study we investigated the combinational effect of PQ1 and tamoxifen. PQ1 has been shown to be a gap junctional activator and induced cell death in both *in vitro* and *in vivo* treatments [26]. Tamoxifen has also shown to induce apoptotic cell death both *in vitro* and *in vivo* [31-33]. This effect was concentration-dependent. At nanomolar (nM) concentrations of tamoxifen, only growth arrest occurs whereas at micromolar (μ M) concentrations induction of cell death was observed in cell cultures [30]. Most of the patients affected with breast cancer receive a daily dose of 20-40 mg tamoxifen [34, 35]. It is difficult to directly translate the dose of tamoxifen from the clinical setting to *in vitro* or *in vivo* studies. However, Mandlekar and Kong showed an IC_{50} of 9-10 μ M in BT-20, MCF-7 and MDA-231 breast cancer cell lines. The results of clinical studies showed that steady state plasma concentrations of tamoxifen can be up to 1 μ M and mean intra-tumor concentrations are even higher, about 4 μ M [30]. We found various concentrations of (1-20 μ M) tamoxifen being used in different cell-based assays [36-38]. Based on the above studies, we chose to observe the effect of 10 μ M tamoxifen in T47D breast cancer cells.

PQ1 and tamoxifen affects cell morphology, proliferation and colony growth in T47D cells

Our previous study showed a tremendous increase in GJIC and caspase 3 activation in the presence of PQ1 in T47D cells [26]. Cell morphology was also affected in the presence of 200 nM PQ1 for 24, 48 and 72 hr in which cells were being detached from the plate. In the presence of 10 μ M tamoxifen for 24, 48, and 72 hr, cells showed a marked change in the morphology, including shrinkage, irregular shape and some cells were floating in the media (Figure 1A, B, C). The combination of PQ1 and tamoxifen showed the complete loss of structure of cells as well. Cells partially regained back their structure in the presence of 10 μ M tamoxifen and 10 nM 17 β -estradiol. These results suggest that the combinational treatment of tamoxifen and PQ1 can potentiate the effect of tamoxifen.

Colony growth assay measures the colony formation in soft agar. Soft agar is used to measure the anchorage-independence (feature of cancerous cells) of the cells. We observed a 55% decrease in the colony growth in the presence of combination of PQ1 and tamoxifen (Figure 2). This suggests that the combinational treatment of tamoxifen and PQ1 is sufficient to cause a significant decrease of colony growth at 7-day incubation compared to tamoxifen or PQ1 treatment alone. Ethanol treatment, a solvent control, shows no effect in colony growth of T47D cells. In MTT assay, 200 and 500 nM PQ1 showed a 60% and 50% cell viability at 48 hr, respectively (Figure 3). Tamoxifen (10 μ M) showed 30% cell viability whereas combination of tamoxifen and PQ1 resulted in only 16% cell viability at 48 hr compared to controls at 48 hr in T47D cells. Both tamoxifen (10 μ M) and PQ1 (200 and 500 nM) resulted in a decrease in cell growth by 50% compared to tamoxifen treatment alone at 48 hr. At 72 hr, combinational treatment of tamoxifen and PQ1 (200 and 500 nM) resulted in 20% and 13% cell viability, respectively. Thus, 48 hr is sufficient to cause a significant decrease with combinational treatment of tamoxifen and PQ1. Interestingly, cell viability was not affected by either 200 or 500 nM PQ1 at 1 hr; however, tamoxifen treatment alone resulted in 28% decrease in cell growth. The cell viability was greatly affected by MTT

assay at 48 hr compared to 72 hr in the presence of PQ1 alone or tamoxifen alone or their combinations.

The expression of proteins in some instances is measured in symptomatic breast cancer to identify the prognostic factors which are associated with the biological behavior of individual tumors. Ki67 is a nuclear protein widely used as a marker for cell proliferation. Tamoxifen has been shown to decrease the expression of Ki67 in breast cancer patients and in breast cancer cell lines [39]. Therefore, the effect of combinational treatment of tamoxifen and PQ1 on Ki67 staining in T47D cells was measured. Ki67 staining was decreased in the presence of 200 nM PQ1 at 24, 48 and 72 hr (Figure 4). In the presence of tamoxifen alone, no Ki67 staining was observed at 24, 48, and 72 hr. Combinational treatment of tamoxifen and PQ1 also showed no Ki67 staining whereas combinational treatment of tamoxifen and estrogen showed an expression of Ki67 suggesting that estrogen can partially antagonize the effect of tamoxifen. These results suggest that PQ1 has an effect on Ki67 staining and it does not antagonize the effect of tamoxifen when used in combination while estrogen can reverse the effect of tamoxifen. Furthermore, PQ1 clearly has an effect on proliferation of T47D cells.

Effect of PQ1 and tamoxifen on apoptotic proteins

Programmed cell death, or apoptosis occurs either by activation of the death receptors or by a breach in the mitochondrial membrane integrity. Cytochrome *c*, a key player in induction of apoptosis cascade, is released from the inside of the mitochondria into the cytosol of the cell by the interaction of two important proteins involved in apoptosis, BAX and Bcl2. Upon apoptotic signals, proapoptotic protein, BAX gets activated while antiapoptotic proteins like Bcl2 prevent apoptosis by heterodimerization with BAX [40, 41]. Overall, the ratio of BAX and Bcl2 determines the integrity of the mitochondrial membrane. In the present study, we conducted a time-dependent study by treating cells with PQ1 and tamoxifen in combination and alone for 1, 48 and 72 hr. We found a significant increase in BAX protein at 1 hr in the presence of 500 nM PQ1 alone, tamoxifen alone, and combination of PQ1 and tamoxifen (Figure 5A). We also observed a decrease in Bcl2 in the presence of tamoxifen alone and in combination of tamoxifen and PQ1 at 1 hr. At 48 hr, a significant decrease was seen in Bcl2 expression at 500 nM PQ1 alone and combination of tamoxifen and PQ1. Also, there was a significant increase in BAX in combinational treatment at of PQ1 (200 and 500 nM) and tamoxifen at 48 hr. Zhang *et al* showed a decrease in Bcl2 but no effect on BAX in MCF-7 cells in the presence of 10 μ M tamoxifen at 72 hr [36]. We found that at 72 hr, BAX and Bcl2 were significantly increased and decreased in the presence of tamoxifen and combination of tamoxifen and either PQ1 200 or 500 nM, respectively. These results implicate that the combinational treatment not only significantly increases BAX but also decreases Bcl2. The ratio of Bcl2 and BAX is decreased significantly at 1 hr in the presence of PQ1 alone and tamoxifen alone or in combination. Therefore, it indicates that PQ1 has a rapid action on BAX activation. The effect on reduction of Bcl2 expression is relatively slow, staying constant at 48 hr before increasing again at 72 hr.

After BAX activation, a cascade of events driven primarily by the activation of proteolytic caspases results in the processing of intracellular structural proteins and regulatory enzymes

that culminates in apoptotic cell death [42]. Caspase 3 activation, an executioner caspase, is considered to be one of the last steps involved in the apoptosis cascade pathway [37]. Therefore, we examined the effect of combination of tamoxifen and PQ1 on caspase 3 activation. Interestingly, we did not find an activation of caspase 3 at 1 hr (Figure 5B). However, there was a significant increase in caspase 3 at 48 and 72 hr in the presence of PQ1 alone, tamoxifen alone and in combination of tamoxifen and PQ1, indicating caspase 3 activation takes time and stays at least through 72 hr. A 50% increase in active caspase 3 was observed at 200 and 500 nM PQ1 at 72 hr. A 2-fold increase was observed in active caspase 3 in the presence of combinational treatment of tamoxifen and PQ1. This suggests that combination of tamoxifen and PQ1 results in increased T47D cell death.

In the present study, we also observed the effect of PQ1 and tamoxifen on an inhibitor of apoptosis (IAP) protein, survivin (a group of proteins involved in inhibition of caspase 3, 7, and 9) [43, 44]. Increase in expression of survivin is believed to protect cells against a possible default induction of apoptosis in the case of aberrant mitosis [45]. Many studies on clinical specimen have shown that survivin expression is invariably upregulated in human cancers and is associated with resistance to chemotherapy linked to poor prognosis, suggesting that survivin modulates the survival of cancer cells [46]. Gazzaniga *et al.* found that survivin cannot be used as a prognostic factor for the relapse of superficial bladder cancer [44]. However, in pre-clinical bladder tumor models, inhibition of survivin expression and/or function has been shown to impede tumor cell proliferation, and markedly induce spontaneous or chemotherapy induced apoptosis [47]. We found no effect on survivin at 1 hr (Figure 5C); however, at 48 and 72 hr we found a tremendous decrease in survivin expression in the treatment of tamoxifen alone and combinational treatment of tamoxifen and PQ1 (200 or 500 nM). This suggests that tamoxifen decreases the survivin expression whereas PQ1 alone has no effect on survivin expression in T47D cells up to 72 hrs. Furthermore, these data were confirmed by confocal microscopy in which a decrease of survivin and BAX was detected (Figure 6A, 6B). The confocal microscopy results showed the absence of survivin in tamoxifen treated cells at 48 and 72 hr.

Measurement of Apoptosis by Nuclear Morphology and Flow Cytometry

The effect of tamoxifen and PQ1 on the nuclear staining by using DAPI was measured. Apoptosis is characterized by morphologic changes such as shrinkage of the cell, condensation of chromatin, and disintegration of the cell into small fragments, apoptotic bodies [48]. In the present study, we found more cells undergoing the process of apoptosis exhibited by blebbing, and fragmentation in the presence of PQ1 and tamoxifen alone and in combination (data not shown). APO-BrdU TUNEL assay kit was used to detect the DNA fragmentation of apoptotic cells. In apoptosis, DNA fragmentation exposes 3'-OH groups at which deoxynucleotidyl transferase (TdT) can add deoxyribonucleotides. 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) is an analog of deoxythymidine which gets incorporated at the 3'-OH group. We found an increase in the BrdUTP incorporation in the presence of PQ1 and tamoxifen alone and in combination at 48 and 72 hr (Figure 7). A 50% increase in apoptotic cells was observed at 48 and 72 hr in the presence of combinational treatment of tamoxifen and PQ1. There was no effect on the apoptosis in the presence of PQ1 or tamoxifen at 1 hr. Even though the apoptosis cascade starts in an hour shown by an

increase in BAX and a decrease in Bcl2, it requires > 1 hr for the activation of caspases and DNA damage. The results imply that the combination of PQ1 (200 and 500 nM) and tamoxifen (10 μ M) results in an increase in apoptosis compared to the individual treatments.

Effect of gap junctional inhibitor, CBX on Cx43, BAX, caspase 3, cyclin D1, MTT assay, Ki67 expression and apoptosis assay by flow cytometry

CBX has been used as a non-specific gap junctional inhibitor in many studies (reference). So far, in our studies we concluded that PQ1 is a gap junctional activator which in the presence of tamoxifen decreases cell proliferation and increases cell death. This can be further confirmed by using a gap junction inhibitor. Therefore, we conducted studies using 20 μ M CBX alone, and in the presence of 10 μ M tamoxifen and 20 μ M CBX. We conducted different time-point western blotting, cell morphology and MTT assay studies and found 20 μ M CBX at 72 hr to be sufficient enough to decrease Cx43, causing no change in cell morphology and non-cytotoxic in T47D cells (data not shown). We performed western blotting of Cx43, BAX, caspase 3 and cyclin D1. We observed that CBX (20 μ M) in T47D cells decreases Cx43, BAX, caspase 3 and increases cyclin D1 expression while combination of tamoxifen and CBX decreased caspase 3 and cyclin D1 expression at 72 hr (Figure 8A). Cyclin D1 is a proto-oncogene and an important regulator of G1 to S-phase transition in numerous cell types from different tissues. Cyclin D1 protein concentration increases as the cell goes into G1 phase and G1-S-phase transition. Our data suggests that gap junction inhibition by CBX causes more cells to enter cell cycle and increases cell proliferation in the presence of CBX as observed by a significant increase in cyclin D1 expression. This effect was observed in the presence of both CBX and tamoxifen further suggesting that the gap junction inhibition decreases the effect of tamoxifen on cell proliferation. However, CBX alone decreased BAX and caspase 3 expression significantly; whereas the combination of CBX and tamoxifen had no significant effect on BAX and caspase 3. This could be either due to a strong tamoxifen effect on cell death or could be due to decreased activity of CBX. MTT assay showed an increase in cell proliferation at CBX alone and combination of CBX and tamoxifen at 72 hr (Figure 8C). However, tamoxifen alone showed a significant decrease in cell proliferation. This suggests that gap junction inhibition affects the cell proliferation, thereby; confirming that gap junctional activators in the presence of tamoxifen can reduce the cell growth. We also observed the effect of CBX on Ki67 expression in T47D cells at 72 hr (Figure 8B). Ki67 was significantly increased in the presence of CBX alone compared to tamoxifen. The combination of tamoxifen and CBX also showed Ki67 expression suggesting that gap junction inhibition affects the cell proliferation. This was also confirmed by cyclin D1 expression and MTT assay. Apoptosis assay showing APO-BrdU incorporation-TUNEL assay showed a decrease in cell death in the presence of CBX; however cell death was not affected in the presence of tamoxifen and CBX (Figure 8D). Tamoxifen alone also caused an increase in cell death compared to control. In the apoptosis assay we used propidium iodide to distinguish between the necrotic and apoptotic cells. The cells which did not incorporate propidium iodide and incorporated APO-BrdU-FITC were counted as apoptotic. This assay, therefore excludes the necrotic cells and allows counting of apoptotic cells. Overall, the data concludes that inhibiting the gap junction by 20 μ M CBX can lead to increased cell proliferation and decrease cell death but a higher dose of CBX is required to decrease cell death in the presence of both

tamoxifen and CBX. This also suggests that gap junctional activators such as PQ1 can decrease cell proliferation and cause cell death which could be a potential therapeutic target in cancerous cells.

Conclusion

Tamoxifen has been the drug of choice for the treatment of endocrine responsive breast tumors, but tamoxifen resistance has been an issue for very long time. We demonstrate for the first time that the combinational effect of PQ1, a gap junction activator, with tamoxifen has a potential use for treatment against breast cancer. We found a decrease in cell proliferation by MTT assay and significant decrease in the colony growth assay. We observed a significant increase in BAX, caspase 3 activation and DNA fragmentation in the presence of combinational treatment of PQ1 and tamoxifen as compared to their individual treatment. We also found that survivin is significantly decreased in the presence of tamoxifen alone and combination of tamoxifen with either 200 or 500 nM PQ1. However, PQ1 alone does not affect survivin expression in T47D cells as observed at 1, 48 and 72 hr. The possibility of PQ1 affecting other caspases in inducing apoptosis has not been covered by the current study; therefore, we cannot rule out the involvement of other caspases. We propose that PQ1 allows tamoxifen (MW= 371.51) to pass through gap junctions between the cells, causing a rapid action of tamoxifen. In support of our data, Jensen and Glazer showed that forced expression of Cx43 in MCF-7 cells resulted in increased cell sensitivity to cisplatin at high density [49]. Our work is demonstrating that the combinational therapy of tamoxifen and PQ1 shows more promising role in inducing apoptosis by caspase 3 activation. Future studies will focus on the combinational effect of PQ1 and tamoxifen *in vivo* as well as reducing tamoxifen concentration in the presence of PQ1.

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Significance

Our study suggests that the combinational treatment allows a decrease in tamoxifen concentration (lower than 10 μ M) in clinical use. This will have a strong implication that combinational treatment with gap junctional activators can lower the concentration of chemotherapeutic agent and thus may reduce side effect of these drugs. In future we would be conducting combinational studies of PQ1 and tamoxifen (dose reduced from μ M to nM) and observe its effect on cell-based systems. Also, much of the work presented has focused in breast cancer cells; however, the role of gap junctional activators (PQ1) in drug sensitivity need not be limited to breast cancer. A variety of other cancers may take advantage of very similar mechanism and as a result other diseases may benefit from gap junction modulating pathway.

Figure 1. Effect of PQ1 and tamoxifen on cell morphology in T47D cells

(A) T47D cells were seeded in a 6-well plate for 24 hr in the presence of 200 nM PQ1, 10 μ M tamoxifen, combination of tamoxifen and PQ1, and the combination of tamoxifen and 10 nM 17β -estradiol (an antagonist for tamoxifen action). (B) Cells were dosed for 48 hrs with the same treatment as above. (C) T47D cells were treated with the same treatment as in (A) for 72 hr. Ethanol was used as a solvent control for tamoxifen at different time intervals. In the presence of PQ1 and tamoxifen alone and in combination the cell structure was seen to be lost significantly at 48 and 72 hrs. At 10 nM 17β -estradiol and tamoxifen, a partial restoration in the cell structure was observed at 48 and 72 hr compared to full restoration at 24 hr.

Figure 2. Combination of 200 nM PQ1 and tamoxifen decreases the colony growth in T47D cells
 Cells were dosed with 200 nM PQ1, 10 µM tamoxifen and combination of both for 7 days in a soft agar. After 7 days, colonies > 50 µm were counted. Combination of PQ1 and tamoxifen resulted in a 55% decrease in the colony growth compared to 20% and 10% decrease in the presence of 200 nM PQ1 and tamoxifen alone, respectively. Graphical representation of three experiments with $\pm$ SD and statistical significance $*p < 0.05$. Note that the significance $*p < 0.05$ was found in all the treatments compared to control at 48 and 72 hr.

Figure 3. MTT assay to measure the T47D cell proliferation in the presence of tamoxifen and PQ1

Cells were treated with 200 and 500 nM PQ1 and 10 µM tamoxifen and combination of tamoxifen and either 200 or 500 nM PQ1 for 1, 48 and 72 hr. At 1 hr, 50 % decrease in cell growth was observed in the presence of tamoxifen and 500 nM PQ1. At 48 hr, PQ1 200 and 500 nM alone, tamoxifen alone, combination of tamoxifen and 200 nM PQ1, and combination of tamoxifen and 500 nM PQ1 showed a cell viability of 60, 50, 30, 16, and 16 %, respectively. At 72 hr the combination of both tamoxifen and either PQ1 200 or 500 nM resulted in 20% and 13% cell viability, showing no further decrease compared to 48 hr. Graphical representation of three experiments with $\pm$ SD and statistical significance $*p < 0.005$ for 48 hr and $**p < 0.05$ for 72 hr. Note the $*p$ values indicate the significance between the tamoxifen treatment alone or with PQ1 combination.

Figure 4. Confocal Microscopy showing the effect of PQ1 and tamoxifen on Ki67 expression
Cells were treated with 200 nM PQ1, 10 μ M tamoxifen, combination of 200nM PQ1 and tamoxifen, and combination of 10 nM 17 β -estradiol for 24, 48 and 72 hr. Ethanol was used as a solvent control for tamoxifen. Cells treated with 200 nM PQ1, tamoxifen or combination of tamoxifen and PQ1 showed a tremendous decrease in Ki67 expression. 17 β -estradiol and tamoxifen partially restored the expression of Ki67.

Figure 5. Expression of proteins involved in apoptosis pathway-BAX, caspase 3, Bcl2 and survivin

(A) Bcl2 and BAX levels were measured in cells were treated with 200 and 500 nM PQ1 and 10 μM tamoxifen and combination of tamoxifen with 200 or 500 nM PQ1 for 1, 48 and 72 hr. A significant increase in BAX was observed at all the treatments compared to control. (B) Caspase 3 expression was measured for the same treatments at 1, 48 and 72 hr. Histogram showing the changes observed in active caspase 3 at 1, 48 and 72 hr with different treatments. A 2-fold increase was seen at 48 and 72 hr in the presence of combination of tamoxifen with 200 and 500nM PQ1. A histogram of three experiments with $\pm$ SD and statistical significance $*p < 0.05$ for 48 hr and $**p < 0.05$ for 72 hr. (C) Survivin measured for the same treatments at 1, 48 and 72 hr. A significant decrease in survivin was observed in the presence of tamoxifen alone and in combination with both 200 and 500nM PQ1 at 48 hr. However, no effect on survivin was seen in the presence of 200 and 500nM PQ1 alone. Actin was used as a loading control for all the proteins.

Figure 6. Confocal microscopy showing the expression of survivin and BAX proteins

(A) Cells were treated with 200 and 500 nM PQ1 and 10 μM tamoxifen and combination of tamoxifen and either 200 or 500 nM PQ1 for 1, 48 and 72 hr. Tamoxifen treatment resulted in a decrease in survivin expression. Cells were stained with antirabbit Alexa fluor 568. (B) Cells were treated the same way as in (A), however; expression of BAX protein was observed using mouse secondary Alexa fluor 488 antibody. Cells were visualized by a confocal microscope (Carl Zeiss LSM 510 META). An increase in BAX was observed in the presence of tamoxifen.

Figure 7. Flow cytometric analysis of apoptotic cells by APO-BrdU labeling

Cells were treated with 200 and 500 nM PQ1 and 10 μM tamoxifen and combination of tamoxifen and 200 or 500 nM PQ1 for 48 and 72 hr. A 50% increase in apoptotic cell number was seen at both 48 and 72 hr in the presence of both tamoxifen and 200 nM or 500 nM PQ1.

Figure 8. Gap junction inhibitor studies using CBX and tamoxifen showing an effect on cell proliferation and cell death in T47D cells at 72 hr

Cells were treated with 10 μM tamoxifen alone, 20 μM CBX alone, and combination of tamoxifen and CBX. Different experiments were performed to confirm the effect of gap junctional activator, PQ1 on cell death by using a gap junction inhibitor, CBX. (A) Western blotting showing Cx43, BAX, caspase 3, and cyclin D1 expression. A significant decrease was observed in Cx43 expression in the presence of CBX. In addition, a significant decrease in BAX, caspase 3 and a significant increase in cyclin D1 was observed in the presence of CBX alone. (B) MTT assay. A histogram of three experiments with $\pm$ SD and statistical significance $*p < 0.05$ for 72 hr in the presence of CBX alone compared to control and $**p < 0.05$ for 72 hr in the presence of combination of CBX and tamoxifen compared to tamoxifen alone. (C) Ki67 expression showed an increase in cell proliferation and increased Ki67 protein expression, respectively. (D) Flow cytometric analysis of apoptotic cells by APO-BrdU labeling.