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

Gunjan Gakhar[a], Duy H. Hua[b] and Thu Annelise Nguyen[a]

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 this study, we tested the efficacy of substituted quinolines (code name = PQ1; a gap junctional activator) in combination with tamoxifen in T47D cells. Colony growth assay was performed using soft agar to measure the colony growth, whereas cell proliferation was measured by the MTT assay in T47D cells. The level of Ki67, survivin, and BAX was measured using confocal microscopy along with western blot analysis. Apoptosis-bromodeoxyuridine triphosphate labeling was also examined in the induced treatment of T47D cells. We observed a 55% decrease in the colony growth in the presence of combination of PQ1 and tamoxifen, whereas tamoxifen alone had little effect. A combination of 10 μmol/l tamoxifen and 200 or 500 nmol/l PQ1 resulted in only 16% cell viability compared with controls at 48 h in T47D cells by the MTT assay. We found a significant increase in BAX protein at 1 h in the presence of 500 nmol/l PQ1 alone, 10 μmol/l tamoxifen alone, and the combination of PQ1 and tamoxifen. A two-fold increase was observed in active caspase 3 in the presence of combinational treatment of 10 μmol/l tamoxifen and 200 or 500 nmol/l PQ1. In addition, flow cytometric analysis showed a 50% increase in the number of apoptotic cells in the presence of the combination of tamoxifen and PQ1 compared with the control. Furthermore, the results show that combinational treatment of tamoxifen and PQ1 significantly reduces the expression of survivin in T47D cells. Gap junction inhibitor studies with carbenexolone were also performed confirming the role of gap junctions in cell proliferation and cell death. The combinational treatment of PQ1 and tamoxifen has a significant increase in BAX expression, caspase 3 activation, and DNA fragmentation. Tamoxifen alone and in combination with PQ1 showed a decrease in the expression of survivin, whereas PQ1 alone was shown to be independent of the 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 with tamoxifen treatment alone. The gap junction inhibitor carbenexolone was shown to increase cell proliferation by increased cyclin D1 expression, MTT assay, and Ki67 expression. It further decreased cell death. This study shows for the first time that combinational treatment of tamoxifen and PQ1 (a gap junctional activator) can be used to potentiate apoptosis of T47D human breast cancer cells. Thus, a gap junctional activator, PQ1, could potentially alter either the length or dose of tamoxifen clinically used for breast cancer patients. *Anti-Cancer Drugs* 21:77–88 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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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 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 have 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 the 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 and tamoxifen, but the results have been controversial [8–13].

Gap junctions are the intercellular plasma membrane channels that allow the passage of small molecules from one cell to other. The flux of molecules through the channels is called the gap junctional intercellular

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communication (GJIC) [14]. GJIC exists in most mammalian cells and is involved in cell growth, differentiation, and homeostasis. It also plays a key role in the occurrence of apoptotic cell death [15]. For decades it has been shown that mitotic cells in the cell cycle show decreased GJIC [16–18]. Therefore, it leads to a state where the cell–cell communication is negatively related to the capability of the cell to grow. On account of its effect on the cell growth, many studies have been conducted to find a correlation between GJIC and cancer. Many in-vitro and in-vivo studies have shown that tumor-promoting agents lead to a decrease in GJIC [19–24]. Protein and mRNA analysis showed a decrease in connexin expression in preneoplastic lesions as well as in hepatocellular carcinomas [25,26].

In a previous study, we reported a new gap junctional activator, a substituted quinoline (code name=PQ1). We showed that PQ1 (200 nmol/l) 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 a 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 [27].

The antitumor effects of tamoxifen are thought to be because of its antiestrogenic activity, mediated by competitive inhibition of estrogen binding to ER and subsequently activation of apoptosis [28,29]. The inhibition of expression of estrogen-regulated genes causes a decrease in cell growth and proliferation [30]. As PQ1 has 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, could further enhance the apoptotic effect in T47D human breast cancer cells. The goal of this study was to examine the effect of combinational treatment of PQ1 and tamoxifen on 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 increased apoptosis by apoptosis–bromodeoxyuridine triphosphate (APO–BrdU) incorporation at different dosing times was observed in the presence of PQ1 and tamoxifen. Furthermore, a decrease in colony growth, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and increased DNA fragmentation occurred in the combinational treatment of PQ1 and tamoxifen. We also conducted inhibitor studies by using carbenoxolone (CBX), a known gap junctional inhibitor [31], alone and in combination with tamoxifen. CBX (20 μmol/l) showed a significant decrease in Cx43, BAX, caspase 3, and a significant increase in cyclin D1. The MTT assay showed a significant increase in cell proliferation; in addition, Ki67 expression was increased in the presence of combination of CBX and tamoxifen. The apoptosis assay by APO–BrdU showed a significant decrease in cell death in the presence of CBX (20 μmol/l) compared with tamoxifen.

Materials and methods

Cell lines and culture

The T47D human breast cancer cell line was purchased from American Type Cell Culture (ATCC, Manassas, Virginia, USA). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia, USA), 10% antibiotic–antimycotic at 37°C with 5% CO_2 in 75-cm^2 flasks. Tamoxifen citrate and carbenexolone were purchased from Sigma (St. Louis, Missouri, USA).

Cell morphology

Cells (5000 cells/ml) were seeded in a six-well plate and dosed with 200 nmol/l PQ1 alone, 10 μmol/l tamoxifen alone, a combination of tamoxifen and PQ1, and a combination of tamoxifen and estrogen (10 nmol/l) for 1, 24, 48, and 72 h. Cells were observed under a microscope using a × 40 objective.

Colony growth using a soft agar assay

Cells were treated with ethanol, 200 nmol/l PQ1, 10 μmol/l tamoxifen, and a combination of 200 nmol/l PQ1 and 10 μmol/l tamoxifen for 7 days. Base agar plates were prepared containing 0.8 and 0.4% agar in RPMI. Cells (5×10^4 cells/33 mm^2 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 assay was performed with cell cultures in a 96-well plate incubated with 200 and 500 nmol/l PQ1, 10 μmol/l tamoxifen alone, and a combination of 10 μmol/l tamoxifen and 200 or 500 nmol/l PQ1 for 1, 48, and 72 h. The gap junction inhibitor studies using 20 μmol/l CBX alone, 10 μmol/l tamoxifen, and a combination of 10 μmol/l tamoxifen and 20 μmol/l CBX were performed at 72 h in T47D cells. The MTT solution was metabolized by the cells (incubation period 1 h) 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 0.35 N HCl solubilization solution, the 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-cm^2 flasks. Cells were incubated with PQ1 alone, 10 μmol/l tamoxifen

alone, and a combination of tamoxifen and PQ1 for 1, 48, and 72 h. For the gap junction inhibition studies, cells were incubated with 20 μmol/l CBX alone, 10 μmol/l tamoxifen, and a combination of 10 μmol/l tamoxifen and 20 μmol/l CBX at 72 h. Cells were washed three times with cold PBS and harvested using cell lysis buffer (20 mmol/l Tris pH 7.5, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 0.5% Triton X-100) with 1 : 1000 dilution of protease inhibitors (Sigma-Aldrich, St. Louis, Missouri, USA) 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. Protein concentration of all the samples was estimated by the Bradford assay using a spectrophotometer. Forty micrograms of whole-cell extract were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Midwest Scientific, St. Louis, Missouri, USA). The nitrocellulose membrane was blocked in 5% milk for 1 h at room temperature and incubated with polyclonal rabbit Bcl2, (1 : 200), mouse BAX (1 : 200; Santa Cruz Biotechnologies, Santa Cruz, California, USA), polyclonal rabbit caspase 3 (1 : 500; BD Pharmingen, San Diego, California, USA), polyclonal rabbit survivin (1 : 1000; Novus Biologicals, Colorado, USA), rabbit cyclin D1 (1 : 1000; Cell Signaling Technology, Danvers, Massachusetts, USA) and polyclonal rabbit actin (1 : 1000; Sigma-Aldrich). Western blots were detected by enhanced chemiluminescence detection (Amersham, Pittsburgh, Pennsylvania, USA).

Immunofluorescence and confocal microscopy

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

Apoptosis assay by flow cytometry

Cells were grown in a 35-mm^2 dish and then dosed with PQ1 alone, tamoxifen alone, and a combination of tamoxifen and PQ1 for 1, 48, and 72 h. Cells were trypsinized and stained with APO–BrdU TUNEL Assay kit (Molecular Probes # A23210, Carlsbad, California, USA) according to the manufacturer's protocol. APO-BrdU binding was analyzed by flow cytometry using a BD FACSCalibur system (BD Biosciences, San Jose, California, USA) and the data obtained were analyzed using the CellQuest software (BD Biosciences). Propidium iodide was used to distinguish necrotic cells from apoptotic cells.

Statistical analysis

The level of significance was considered at a P value less than 0.05 using Student's t-test analysis. All experiments were performed at least three times individually and are presented as mean ± SD.

Results and discussion

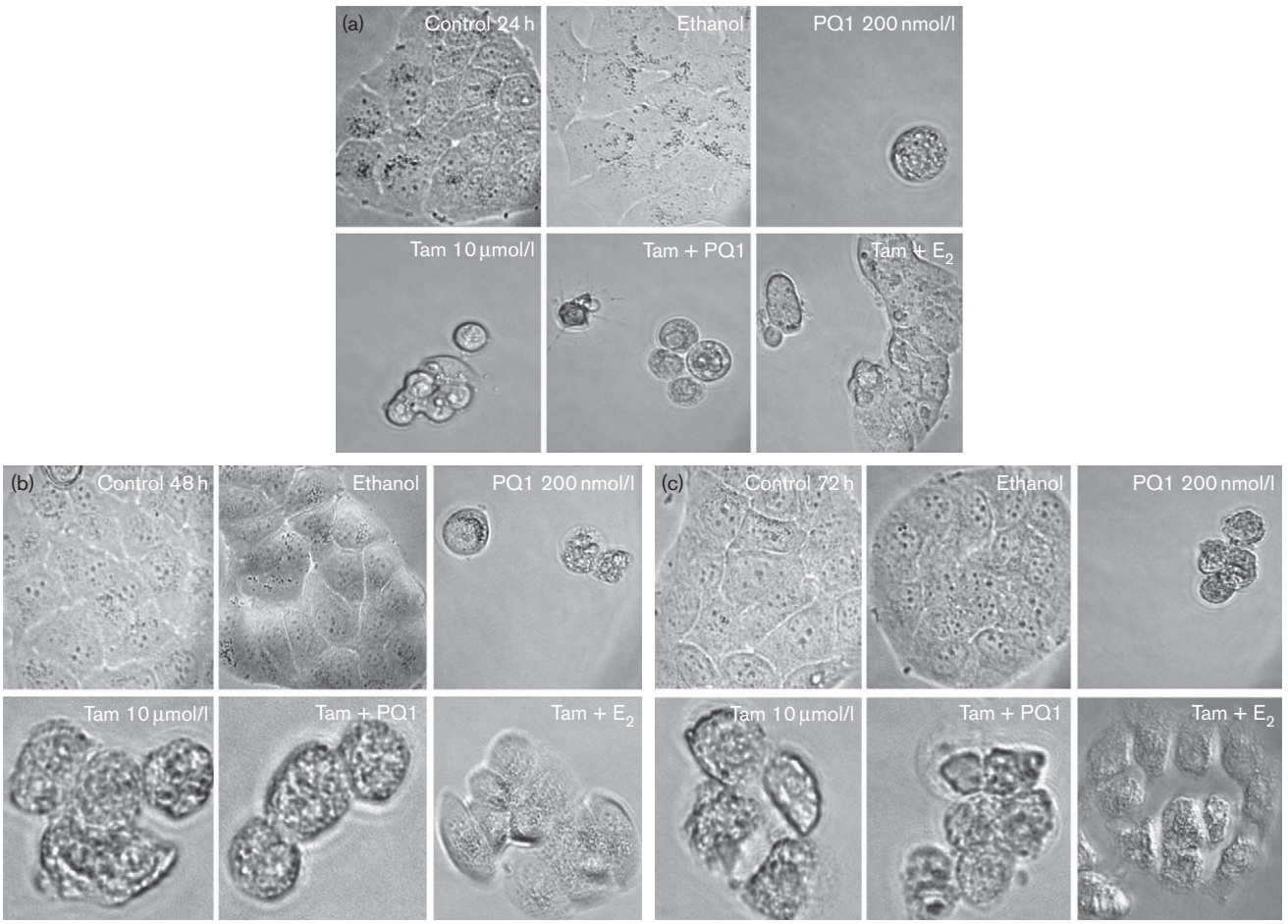
Breast cancer is the second leading cause of cancer deaths in North American women. Tamoxifen has been the single agent of choice in treating hormone-responsive breast cancer cases since 1971. Although tamoxifen has been shown to be 50% more effective than placebo in preventing the occurrence of breast cancer in high-risk populations, the risk of developing uterine cancer has increased by more than 40% [32]. The line of treatment with tamoxifen includes using 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 this 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 [27]. Tamoxifen has also shown to induce apoptotic cell death both *in vitro* and *in vivo* [33–35]. This effect was concentration dependent. At nanomolar (nmol/l) concentrations of tamoxifen, only growth arrest occurs, whereas at micromolar (μmol/l) concentrations induction of cell death was observed in cell cultures [32]. Most of the patients affected with breast cancer receive a daily dose of 20–40 mg tamoxifen [36,37]. 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 [32] showed an IC_{50} of 9–10 μmol/l 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 μmol/l and mean intratumor concentrations are even higher, about 4 μmol/l. We found various concentrations of (1–20 μmol/l) tamoxifen being used in different cell-based assays [38–40]. On the basis of the above studies, we chose to observe the effect of 10 μmol/l tamoxifen in T47D breast cancer cells.

PQ1 and tamoxifen affect 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 [27]. Cell morphology was also affected in the presence of 200 nmol/l PQ1 for 24, 48, and 72 h in which cells were being detached from the plate. In the presence of 10 μmol/l tamoxifen for 24, 48, and 72 h, cells showed a marked change in the morphology, including shrinkage, irregular shape, and some cells floating in the media (Fig. 1a–c). However, no effect in cell morphology was observed at 1 h in the presence of 200 and 500 nmol/l PQ1 alone and in combination with 10 μmol/l tamoxifen (data not shown). The combination of PQ1 and tamoxifen showed the complete loss of structure of cells as well. Cells partially regained their structure in the presence of 10 μmol/l tamoxifen and 10 nmol/l 17β-estradiol. These results suggest that the combinational treatment of tamoxifen and PQ1 can potentiate the effect of tamoxifen.

The colony growth assay measures the colony formation in soft agar, which is used to measure the anchorage independence (a feature of cancerous cells) of the cells. We observed a 55% decrease in colony growth in the presence of the combination of PQ1 and tamoxifen (Fig. 2). This suggests that the combinational treatment of tamoxifen and PQ1 is sufficient to cause a significant decrease in colony growth at 7-day incubation compared with tamoxifen or PQ1 treatment alone. Ethanol treatment, a solvent control, shows no effect on colony growth of T47D cells. In the MTT assay, 200 and 500 nmol/l PQ1 showed 60 and 50% cell viability at 48 h, respectively (Fig. 3). Tamoxifen (10 μmol/l) showed 30% cell viability, whereas the combination of tamoxifen and PQ1 resulted in only 16% cell viability at 48 h compared with controls at

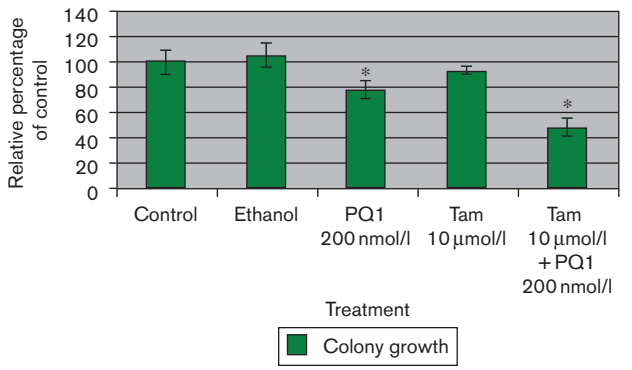
Fig. 1



Effect of PQ1 and tamoxifen (Tam) on cell morphology in T47D cells. (a) T47D cells were seeded in a six-well plate for 24 h in the presence of 200 nmol/l PQ1, 10 μmol/l Tam, the combination of Tam and PQ1, and the combination of Tam and 10 nmol/l 17β-estradiol (an antagonist for Tam action). (b) Cells were dosed for 48 h with the same treatment as above. (c) T47D cells were treated with the same treatment as in (a) for 72 h. Ethanol was used as a solvent control for Tam at different time intervals. In the presence of PQ1 and Tam alone and in combination, the cell structure was seen to be lost significantly at 48 and 72 h. At 10 nmol/l 17β-estradiol and Tam, a partial restoration in the cell structure was observed at 48 and 72 h compared with full restoration at 24 h.

48 h in T47D cells. Both tamoxifen (10 μmol/l) and PQ1 (200 and 500 nmol/l) resulted in a decrease in cell growth by 50% compared with tamoxifen treatment alone at 48 h. At 72 h, combinational treatment of tamoxifen and PQ1 (200 and 500 nmol/l) resulted in 20 and 13% cell viability, respectively. Thus, 48 h 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 nmol/l PQ1 at 1 h; however, tamoxifen treatment alone resulted in a 28% decrease in cell growth. The cell viability was greatly affected by the MTT assay at 48 h compared with 72 h in the presence of PQ1 alone or tamoxifen alone or their combinations.

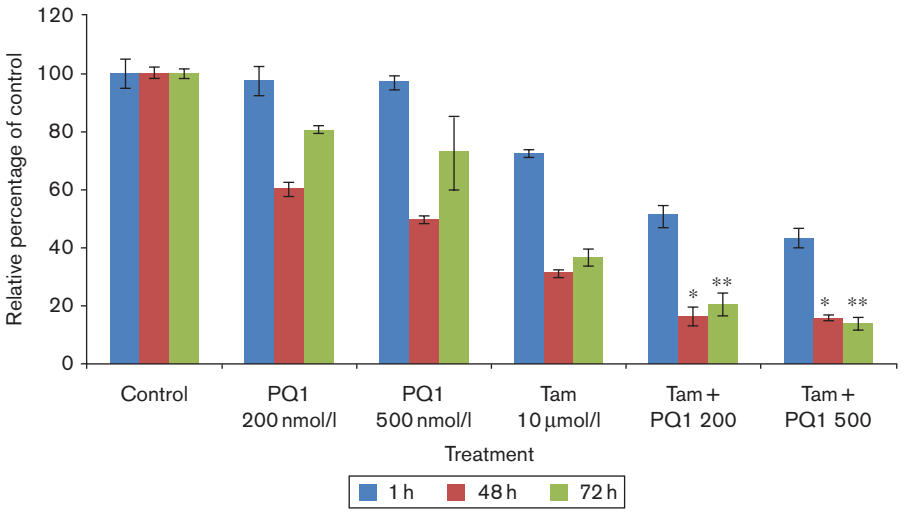
Fig. 2



Combination of 200 nmol/l PQ1 and tamoxifen (Tam) decreases the colony growth in T47D cells. Cells were dosed with 200 nmol/l PQ1, 10 μmol/l Tam, and combination of both for 7 days in a soft agar. After 7 days, colonies greater than 50 μm were counted. Combination of PQ1 and Tam resulted in a 55% decrease in the colony growth compared with 20 and 10% decrease in the presence of 200 nmol/l PQ1 and Tam alone, respectively. Graphical representation of three experiments with ± SD and statistical significance $*P<0.05$.

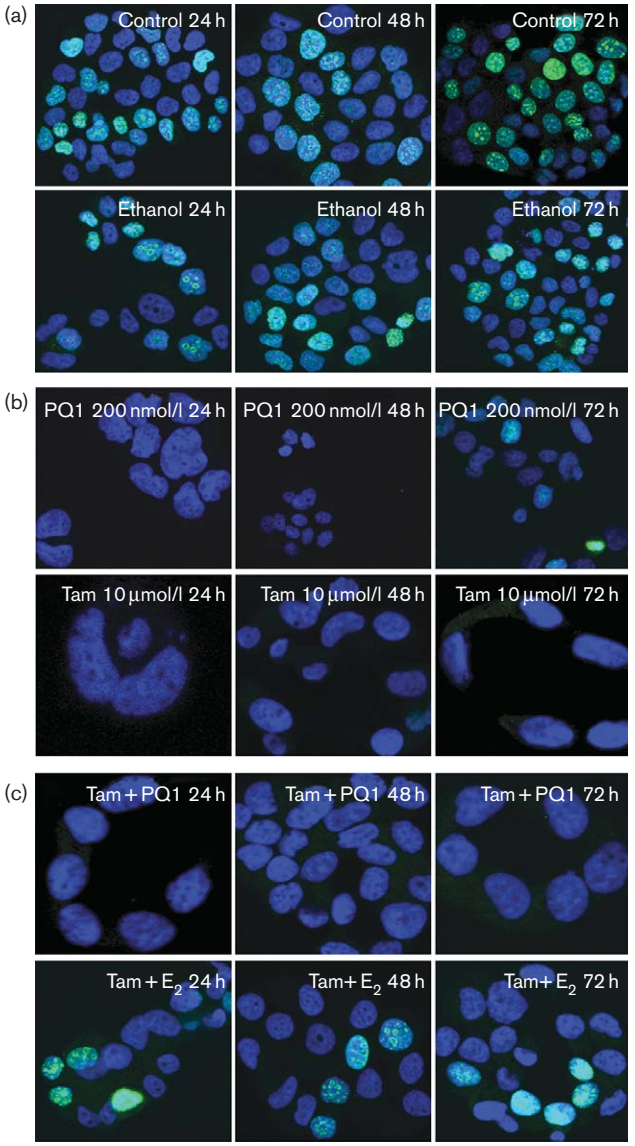
The expression of proteins in some instances is measured in symptomatic breast cancer to identify the prognostic factors, which are associated with the biological behavior of individual tumors. Ki67 is a nuclear protein widely used as a marker for cell proliferation. Tamoxifen has been shown to decrease the expression of Ki67 in breast cancer patients and in breast cancer cell lines [41]. 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 nmol/l PQ1 at 24, 48, and 72 h (Fig. 4). In the presence of tamoxifen alone, no Ki67 staining was observed at 24, 48, and 72 h. 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.

Fig. 3



MTT assay to measure the T47D cell proliferation in the presence of tamoxifen (Tam) and PQ1. Cells were treated with 200 and 500 nmol/l PQ1, 10 μmol/l Tam, and a combination of Tam and either 200 or 500 nmol/l PQ1 for 1, 48, and 72 h. At 1 h, 50% decrease in cell growth was observed in the presence of Tam and 500 nmol/l PQ1. At 48 h, 200 and 500 nmol/l PQ1 alone, Tam alone, the combination of Tam and 200 nmol/l PQ1, and the combination of Tam and 500 nmol/l PQ1 showed a cell viability of 60, 50, 30, 16, and 16%, respectively. At 72 h, the combination of both Tam and either PQ1 200 or 500 nmol/l resulted in 20 and 13% cell viability, showing no further decrease compared with 48 h. Graphical representation of three experiments with ± SD and statistical significance $*P<0.005$ for 48 h and $**P<0.05$ for 72 h. Note the $*P$ values indicate the significance between the Tam treatment alone or with PQ1 combination.

Fig. 4



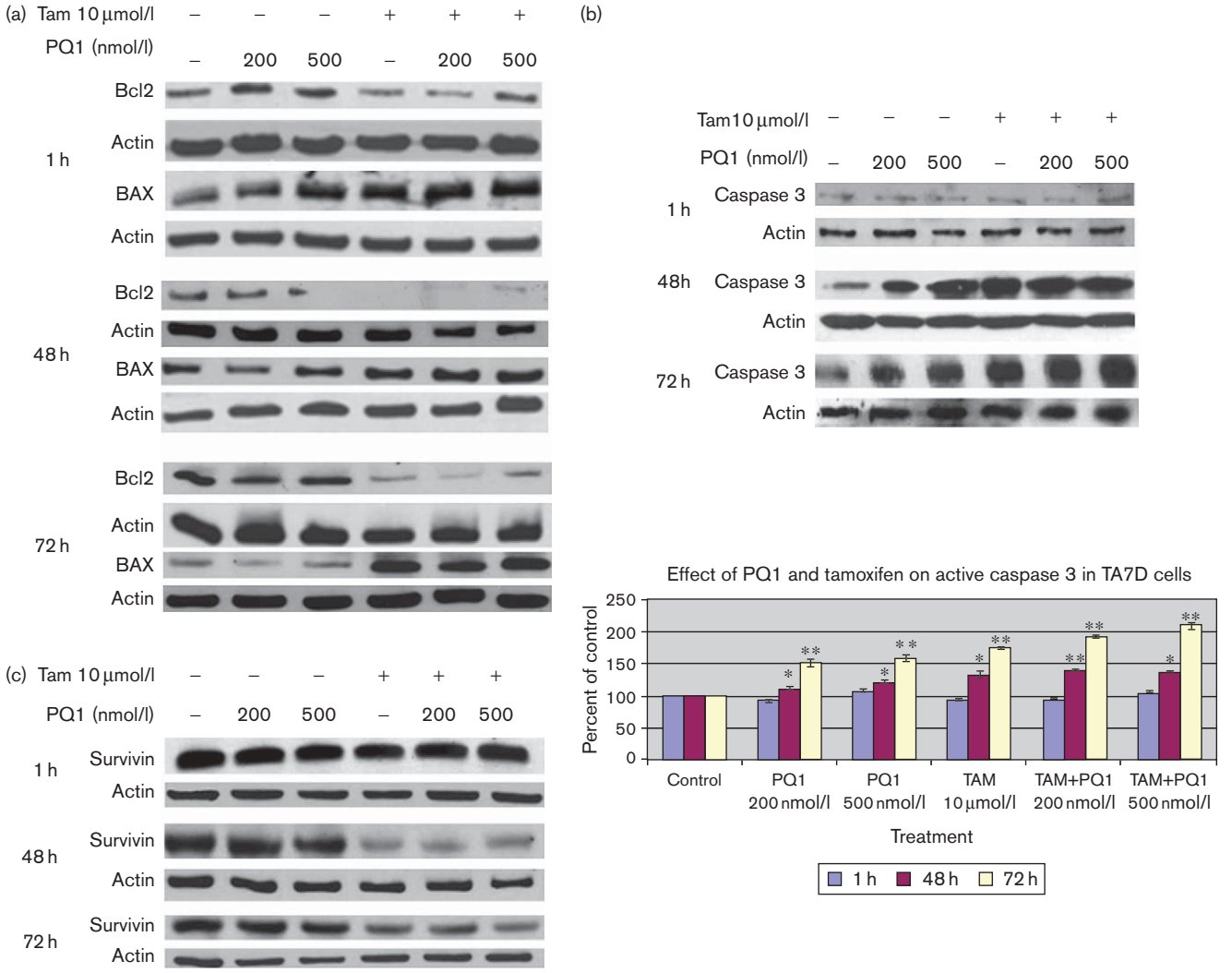
(a) Ki67 expression in untreated cells serving as a control and cells treated with ethanol [solvent for tamoxifen (Tam)] at 24, 48, and 72 h. We found no effect on the Ki67 in the presence of ethanol at different time periods. (b) Ki67 expression was observed at varying concentrations of PQ1 and Tam at 24, 48, and 72 h. We found a significant decrease in the Ki67 expression in the presence of both PQ1 and Tam. However, at 72 h, Ki67 expression was observed in the presence of PQ1. (c) Ki67 expression in the presence of combination of PQ1 and Tam, and combination of Tam and estrogen. Ki67 expression was decreased significantly in the presence of both PQ1 and Tam at 24, 48, and 72 h. Estrogen was used to act as an antagonist to Tam. We found that Ki67 was reexpressed in the presence of both Tam and estrogen.

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 the induction of the apoptosis cascade, is released from inside the mitochondria into the cell cytosol by the interaction of two important proteins involved in apoptosis, BAX and Bcl2. Upon apoptotic signals, proapoptotic protein, BAX, is activated, whereas antiapoptotic proteins, such as Bcl2, prevent apoptosis by heterodimerization with BAX [42,43]. Overall, the ratio of BAX and Bcl2 determines the integrity of the mitochondrial membrane. In this study, we conducted a time-dependent study by treating cells with PQ1 and tamoxifen in combination and alone for 1, 48, and 72 h. We found a significant increase in BAX protein at 1 h in the presence of 500 nmol/l PQ1 alone, tamoxifen alone, and combination of PQ1 and tamoxifen (Fig. 5a). We also observed a decrease in Bcl2 in the presence of tamoxifen alone and in the combination of tamoxifen and PQ1 at 1 h. At 48 h, a significant decrease was observed in Bcl2 expression at 500 nmol/l PQ1 alone and the combination of tamoxifen and PQ1. Furthermore, there was a significant increase in BAX in the combinational treatment using PQ1 (200 and 500 nmol/l) and tamoxifen at 48 h. Zhang *et al.* [38] showed a decrease in Bcl2 but no effect on BAX in MCF-7 cells in the presence of 10 µmol/l tamoxifen at 72 h. We found that at 72 h, BAX and Bcl2 were significantly increased and decreased in the presence of tamoxifen and the combination of tamoxifen and either 200 or 500 nmol/l PQ1, respectively. These results indicate that the combinational treatment not only significantly increases BAX but also decreases Bcl2. The ratio of Bcl2 and BAX is decreased significantly at 1 h 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, remaining constant at 48 h before increasing again at 72 h.

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 culminate in apoptotic cell death [44]. Caspase 3 activation, an executioner caspase, is considered to be one of the last steps involved in the apoptosis cascade pathway [39]. Therefore, we examined the effect of the combination of tamoxifen and PQ1 on caspase 3 activation. Interestingly, we did not find any activation of caspase 3 at 1 h (Fig. 5b). However, there was a significant increase in caspase 3 at 48 and 72 h in the presence of PQ1 alone, tamoxifen alone, and with the combination of tamoxifen and PQ1, indicating that caspase 3 activation takes time and remains at least through 72 h. A 50% increase in active caspase 3 was observed at 200 and 500 nmol/l PQ1 at 72 h. A two-fold increase was observed in active caspase 3 in the presence of combinational treatment of tamoxifen and PQ1. A histogram of three experiments with ± SD and statistical significance $P < 0.05$ for 48 h and $P < 0.05$ for 72 h for caspase 3 provides more detailed information (Fig. 5b). The densitometric analysis shows that caspase 3 significantly increases at 48 and 72 h in the presence of 200 and

Fig. 5

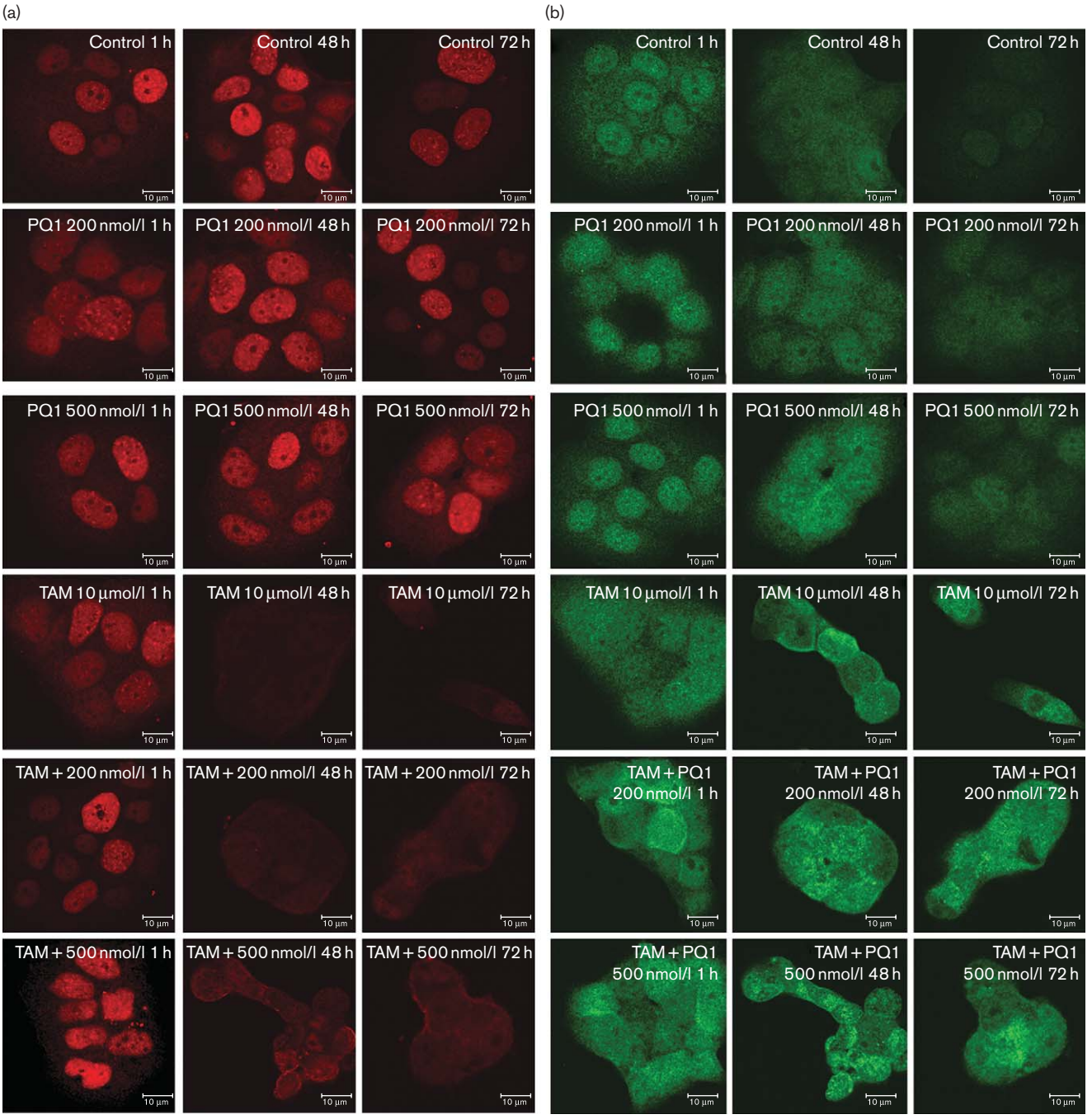


Expression of proteins involved in apoptosis pathway – BAX, caspase 3, Bcl2, and survivin. (a) Bcl2 and BAX levels were measured in cells were treated with 200 and 500 nmol/l PQ1 and 10 µmol/l tamoxifen (Tam) and a combination of Tam with 200 or 500 nmol/l PQ1 for 1, 48, and 72 h. A significant increase in BAX was observed at all the treatments compared with control. (b) Caspase 3 expression was measured for the same treatments at 1, 48, and 72 h. Histogram showing the changes observed in active caspase 3 at 1, 48, and 72 h with different treatments. A two-fold increase was seen at 48 and 72 h in the presence of the combination of Tam with 200 and 500 nmol/l PQ1. A histogram of three experiments with ±SD and statistical significance $*P<0.05$ for 48 h and $**P<0.05$ for 72 h. (c) Survivin measured for the same treatments at 1, 48, and 72 h. A significant decrease in survivin was observed in the presence of Tam alone and in combination with both 200 and 500 nmol/l PQ1 at 48 h. However, no effect on survivin was seen in the presence of 200 and 500 nmol/l PQ1 alone. Actin was used as a loading control for all the proteins.

500 nmol/l PQ1 and in combination with tamoxifen, compared with their respective controls. This suggests that the combination of tamoxifen and PQ1 results in increased T47D cell death.

In this study, we also observed the effect of PQ1 and tamoxifen on an inhibitor of apoptosis protein, survivin (a group of proteins involved in inhibition of caspases 3, 7, and 9) [45,46]. Increase in the expression of survivin is believed to protect cells against a possible default induction of apoptosis in the case of aberrant mitosis [47]. Many studies on clinical specimens 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 [48]. Gazzaniga *et al.* [46] found that survivin cannot be used as a prognostic factor for the relapse of superficial bladder cancer. However, in preclinical 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 [49]. We found no effect on survivin at 1 h (Fig. 5c); however, at 48 and 72 h we found a tremendous decrease in survivin expression in the treatment using tamoxifen alone and the combinational treatment of tamoxifen and

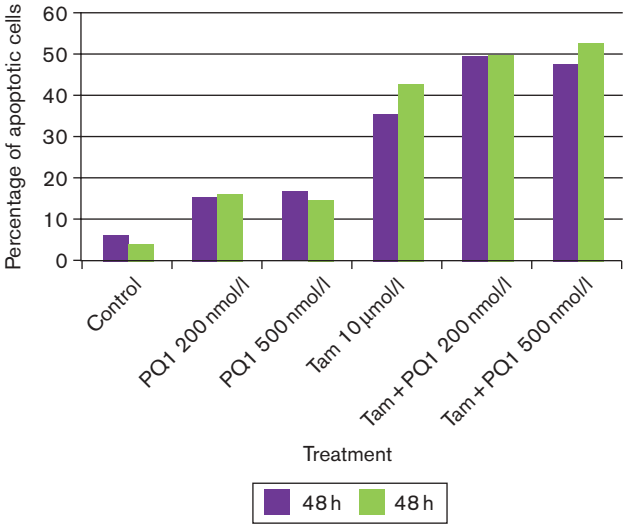
Fig. 6



Confocal microscopy showing the expression of survivin and BAX proteins. (a) Cells were treated with 200 and 500 nmol/l PQ1 and 10 µmol/l Tam and a combination of Tam and either 200 or 500 nmol/l PQ1 for 1, 48 and 72 h. Tam 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 Tam.

PQ1 (200 or 500 nmol/l). This suggests that tamoxifen decreases survivin expression, whereas PQ1 alone has no effect on survivin expression in T47D cells up to 72 h. Furthermore, these data were confirmed by confocal microscopy in which a decrease of survivin and BAX was detected (Fig. 6a and b). The confocal microscopy results showed the absence of survivin in tamoxifen-treated cells at 48 and 72 h.

Fig. 7



Flow cytometric analysis of apoptotic cells by APO-BrdU labeling. Cells were treated with 200 and 500 nmol/l PQ1 and 10 µmol/l tamoxifen (Tam) and a combination of Tam and 200 or 500 nmol/l PQ1 for 48 and 72 h. A 50% increase in the number of apoptotic cells was seen at both 48 and 72 h in the presence of both Tam and 200 or 500 nmol/l PQ1.

Measurement of apoptosis by nuclear morphology and flow cytometry

The effect of tamoxifen and PQ1 on nuclear staining was measured by using DAPI. Apoptosis is characterized by morphological changes such as shrinkage of the cell, condensation of chromatin, and disintegration of the cell into small fragments (apoptotic bodies) [50]. In this 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). The 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 can add deoxyribonucleotides. 5-Bromo-2′-deoxyuridine 5′-triphosphate is an analog of deoxythymidine, which is incorporated at the 3′-OH group. We found an increase in the incorporation of 5-bromo-2′-deoxyuridine 5′-triphosphate in the presence of PQ1 and tamoxifen alone and in combination at 48 and 72 h (Fig. 7). A 50% increase in apoptotic cells was observed at 48 and 72 h in the presence of combinational treatment of tamoxifen and PQ1. There was no effect on apoptosis in the presence of PQ1 or tamoxifen at 1 h. Although the apoptosis cascade starts in an hour shown by an increase in BAX and a decrease in Bcl2, it requires more than 1 h for the activation of caspases and DNA damage. The results imply that the combination of PQ1 (200 and 500 nmol/l) and tamoxifen (10 µmol/l) results in an increase in apoptosis compared with the individual treatments.

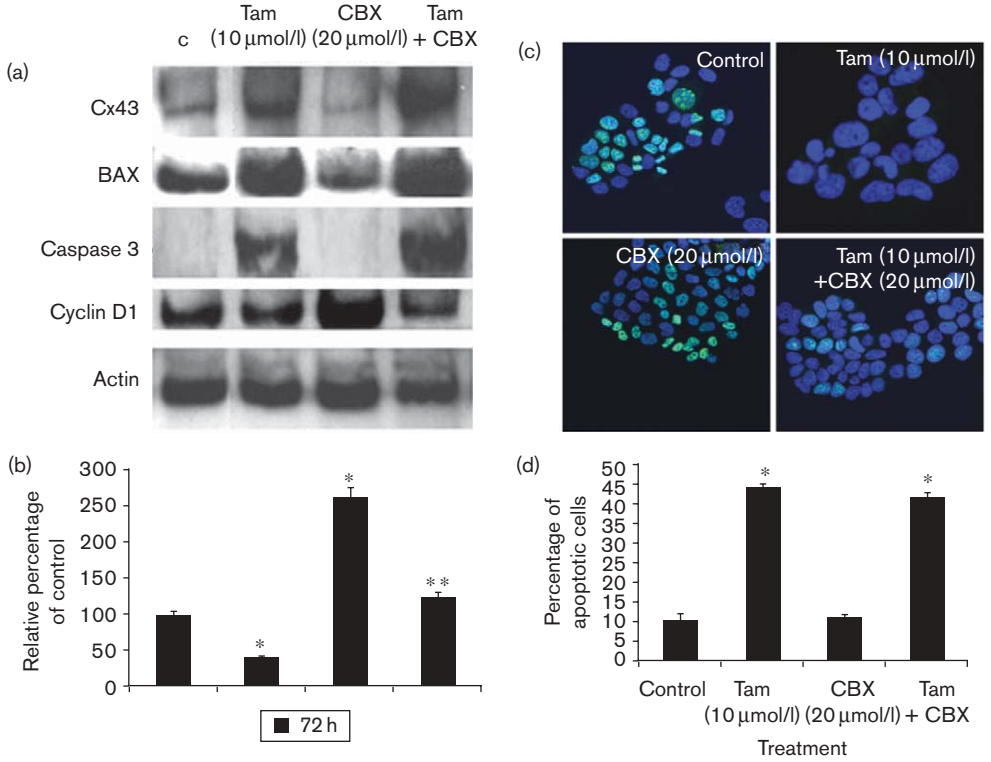
Effect of the gap junctional inhibitor, CBX, on Cx43, BAX, caspase 3, and cyclin D1

CBX has been used as a nonspecific gap junctional inhibitor in many studies. 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 further confirm the important role of the gap junction in cell proliferation through experiments using a gap junctional inhibitor. Therefore, we conducted studies using 20 µmol/l CBX alone, and in the presence of 10 µmol/l tamoxifen and 20 µmol/l CBX. We conducted different timepoint western blotting, cell morphology, and MTT assay studies and found 20 µmol/l CBX at 72 h to be sufficient to decrease Cx43, causing no change in cell morphology and noncytotoxic in T47D cells (data not shown). We performed western blotting of Cx43, BAX, caspase 3, and cyclin D1. We observed that CBX (20 µmol/l) in T47D cells decreases Cx43, BAX, and caspase 3 and increases cyclin D1 expression, whereas the combination of tamoxifen and CBX decreased caspase 3 and cyclin D1 expression at 72 h (Fig. 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 [51]. Our data suggest that gap junction inhibition by CBX causes more cells to enter the 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 gap junction inhibition decreases the effect of tamoxifen on cell proliferation. 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 because of a strong tamoxifen effect on cell death or a result of decreased activity of CBX.

Effect of CBX on MTT assay, Ki67 expression, and apoptosis assay by flow cytometry

MTT assay showed an increase in cell proliferation at CBX alone and combination of CBX and tamoxifen at 72 h (Fig. 8c). However, tamoxifen alone showed a significant decrease in cell proliferation. This suggests that gap junction inhibition affects cell proliferation, thereby confirming that gap junctional activators in the presence of tamoxifen can reduce cell growth. We also observed the effect of CBX on Ki67 expression in T47D cells at 72 h (Fig. 8b). Ki67 was significantly increased in the presence of CBX alone compared with tamoxifen. The combination of tamoxifen and CBX also showed Ki67 expression, indicating that gap junction inhibition affects cell proliferation. This was also confirmed by cyclin D1 expression and MTT assay. Apoptosis assay showing APO–BrdU incorportation-TUNEL assay showed a decrease in cell death in the presence of CBX; however, cell

Fig. 8



Gap junction inhibitor studies using CBX and tamoxifen (Tam) showing an effect on cell proliferation and cell death in T47D cells at 72 h. Cells were treated with 10 μmol/l Tam alone, 20 μmol/l CBX alone, and combination of Tam 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 ± SD and statistical significance $*P<0.05$ for 72 h in the presence of CBX alone compared with control and $**P<0.05$ for 72 h in the presence of combination of CBX and Tam compared with Tam 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.

death was not affected in the presence of tamoxifen and CBX (Fig. 8d). Tamoxifen alone also caused an increase in cell death compared with the control. In the apoptosis assay, we used propidium iodide to distinguish between necrotic and apoptotic cells. Cells that did not incorporate propidium iodide but incorporated APO–BrdU–fluorescein isothiocyanate were counted as apoptotic. Therefore, this assay excludes necrotic cells and allows the amount of apoptotic cells to be measured. Overall, the data lead to the conclusion that inhibiting the gap junction by using 20 μmol/l CBX can increase 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. These data regarding gap junctional inhibitors suggest that gap junctional activators such as PQ1 can be used as therapeutic targets in cancerous cells in which gap junctional activators can decrease cell proliferation and cause cell death.

Conclusion

Tamoxifen has been the drug of choice for the treatment of endocrine-responsive breast tumors, but tamoxifen resistancehas been an issue for very long time. We show for the first time that the combinational effect of PQ1, a gap junctional activator, with tamoxifen has a potential use for treatment against breast cancer. We found a decrease in cell proliferation by the MTT assay and a 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 with their individual treatment. We also found that survivin is significantly decreased in the presence of tamoxifen alone and a combination of tamoxifen with either 200 or 500 nmol/l PQ1. However, PQ1 alone does not affect survivin expression in T47D cells as observed at 1, 48, and 72 h. The gap junction inhibitor studies conducted by using 20 μmol/l CBX further confirm that gap junctions play a significant role in cell proliferation and cell death. Furthermore, the possibility of PQ1 affecting other caspases in inducing apoptosis has not been covered by this study; therefore, we cannot rule out the involvement of other caspases. We propose that PQ1 allows tamoxifen (molecular weight=371.51) to pass through gap junctions

between the cells, causing rapid action of tamoxifen. In support of our data, Jensen and Glazer [52] showed that forced expression of Cx43 in MCF-7 cells resulted in increased cell sensitivity to cisplatin at high density. Our work is showing that combinational therapy with tamoxifen and PQ1 shows a 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 the tamoxifen concentration in the presence of PQ1.

Significance

Our study suggests that the combinational treatment allows a decrease in tamoxifen concentration (lower than 10 µmol/l) 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 effects of these drugs. However, in a clinical setting, many factors are involved and the outcomes cannot be completely predicted. In addition, pharmacokinetic studies on PQ1 could shed more light on the possible effect of PQ1 in a combinational clinical trial. In future we would be conducting combinational studies of PQ1 and tamoxifen (dose reduced from µmol/l to nmol/l) and observe its effect on cell-based systems. Much of the work presented has focused on 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 a very similar mechanism and as a result other diseases may benefit from gap junction modulating pathways.

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