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INVESTIGATION OF THE ROLES OF ATM AND ATR IN TAMOXIFEN-INDUCED APOPTOSIS IN 4T1 MOUSE BREAST CANCER CELLS

NAFIS HASAN[¥], ROBERT KURT[†], PH.D AND SHYAMAL K. MAJUMDAR*, PH.D.,

[¥]Laboratory Assistant, Thomas Jefferson University,
[†]Associate Professor, *Emeritus Kreider Professor (corresponding author), Biology Department,
Lafayette College, Easton, PA 18042 USA

ABSTRACT

Tamoxifen has long been used as a treatment for advanced and metastatic estrogen receptor positive breast cancer and also for reducing incidences of breast cancer in high-risk populations. Tamoxifen exerts its effects as a selective estrogen receptor modulator (SERM), thus binding to estrogen receptors and inhibiting estrogen activity. But this activity, along with the genotoxic effects of Tamoxifen, results in apoptosis and necrosis of both cancerous and healthy cells. This gives rise to the well-characterized side effects of Tamoxifen such as uterine and endometrial cancers. The major mechanisms of apoptosis by Tamoxifen have already been described in literature, but the ATR-ATM-TP53 pathway has not been reported. The purpose of this study was to investigate the roles of ATR and ATM in Tamoxifen-induced apoptosis of estrogen receptor positive (ER(+)) 4T1 mouse breast cancer cells. The study employs cell growth assays with differential Tamoxifen treatments, silencing of ATR and ATM genes using RNA interference, gene expression analysis using qPCR and also uses post-translational caspase-9 and p38 inhibitors to accomplish its goals. The results of the study show that inhibition of ATR and ATM genes did not significantly increase cell viability in presence of Tamoxifen, contrary to the initial assumptions. This strongly suggests that ATR and ATM do not play a significant role in the Tamoxifen-induced apoptosis of ER(+) 4T1 cells.

Keywords – Tamoxifen, DNA damage, breast cancer, ataxia telangiectasia, mutated (ATM), ATM and Rad3-related (ATR), apoptosis.

INTRODUCTION

Tamoxifen ((Z)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-N,N,dimethylethanamine) has been used for the treatment of breast cancer since its approval by the FDA in 1977 (Friedman 1998; Wozniak et al 2007). Since then, much work has been focused on its mechanism of action and it has been elucidated that it mainly acts as a Selective Estrogen Receptor Modulator (SERM), and inhibits estrogen activity by competitively binding with the estrogen receptor (ER), especially ERα (Jordan 2004). It has also been shown to be toxic to both cancerous and non-cancerous cells, leading to apoptosis and necrosis at high concentrations (Favara et al 2008; Goel et al 2008; Honorat et al 2008; Wozniak et al 2007). The apoptotic action has been tied to various intrinsic pathways that involve: (i) calmodulin (calcium modulated protein that binds calcium), (ii) c-Jun N-terminal Kinase (JNK)/p38 pathway, (iii) generation of ceramides (family of sphingolipid molecules that act as proapoptotic molecules), (iv) mitochondrial caspases, (v) phospholipase C or D (PLC/D –

enzymes that cleave phospholipids resulting in increase in calcium concentration in the cell), (vi) protein kinase C (PKC, which is induced by Tamoxifen to trigger cytochrome c release that initiates apoptosis), (vii) c-Myc (transcription factor required for apoptosis), and (viii) transforming growth factor β (TGFβ, an inhibitory cytokine) (Mandlekar and Kong 2001; Obrero et al 2002; Salami and Karami-Tehrani 2003).

Recent studies have linked Tamoxifen usage to increased incidences of endometrial and uterine cancers. These side effects are caused by both its action as a SERM and its genotoxicity (deleterious effects on a cell's genetic material), especially in high concentrations (Jordan 2004; Phillips 2001; Poirier and Schild 2003; Smith and Brown 2000; Wozniak et al 2007). In vitro assays and studies in mouse and rat models have shown Tamoxifen to cause DNA adducts, single and double-stranded breaks in the DNA through generation of free radical species, and chromosomal breaks (Favara et al 2008; Martin et al 1997; Mizutani et al 2004; Phillips 2001; Poirier and Schild 2003; Wozniak et al 2007). Treatment with Tamoxifen has also resulted in tumors in testes, ovaries and uterus in mouse models (Phillips 2001); in case of humans, relatively high levels of DNA adducts have been detected in endometrial tissues of women undergoing Tamoxifen treatment, indicating Tamoxifen's involvement in increased incidences of endometrial and uterine cancers (Shibutani et al 1999; Shibutani et al 2000).

MATERIALS & METHODS

1. Cell Lines & Maintenance. Mouse breast cancer 4T1 cells (estrogen receptor positive - ER(+)) were grown and maintained in Dulbecco's Modified Eagle Medium with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (DME-10) at 37°C in a humidified incubator with 7.5% CO_2 in air. For reculturing, cells were harvested by trypsinization and two different cell cultures were made at 1:4 (1 ml cell solution + 4 ml DME-10) and 0.5:4 (0.5 ml cell solution + 4 ml DME-10) concentrations. Trypsinization was performed as following – cell

The apoptosis resulting from DNA damage in such cases is similar to that caused by cisplatin, a broad-spectrum anti-cancer drug that induces renal cell death through apoptosis (Pabla et al 2008). This mechanism is mediated through ATR-CHEK2 (CHK2 checkpoint homolog) signaling downstream of p53 (Tumor suppressor protein 53/ TP53), which initiates cell cycle arrest and then apoptosis (Pabla et al 2008). The proteins ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) are two key DNA damage sensing proteins that are responsible for initiating the cell cycle signaling cascade (Brown and Baltimore 2003; Canman et al 1998; Morgan 2007; Sancar et al 2004; Zou and Elledge 2003). ATR is usually involved in sensing replication stress and single-stranded DNA damage and its activation results in cell cycle arrest (Brown and Baltimore 2003; Sancar et al 2004; Zou and Elledge 2003). ATM is activated in the case of double-stranded breaks and the response may either be a rapid cell cycle arrest or a delayed cell death through apoptosis (Canman et al 1998; Morgan 2007). Both these proteins ultimately lead to the activation of p53 that results in either cell cycle arrest or apoptosis (Fridman and Lowe 2003; Morgan 2007). Consequently, it has been shown that estrogen inhibits ATR signaling to cell cycle checkpoints (Pedram et al 2007). That, combined with Tamoxifen's genotoxicity, provides the aim for this research, which was to investigate the roles of ATR and ATM in apoptosis of 4T1 mouse breast cancer cells following Tamoxifen treatment.

culture supernatant was removed and the flask was washed with 1 ml of Phosphate Buffered Solution without calcium/magnesium solution (PBS). Trypsin-EDTA (2 ml) was added to flask and the cells were observed under light microscope for dislodging. After sufficient cells were in suspension, trypsin action was inhibited by addition of 2 ml DME-10 and the mixture was carefully aspirated and transferred to a 15 ml centrifuge tube. The flask was washed with additional 2 ml DME-10 and solution was transferred to the same tube. The tube

was centrifuged for 8 minutes at 725 X g and cell pellet was resuspended in 3.0 ml DME-10. Cells were recultured every 48 hours to maintain robust growth at 70% confluency.

2. Cell Count. Cell counts using the Vi-cell® cell viability analyzer (Beckman Coulter, Brea, CA) were performed by harvesting cells through trypsinization and transferring 1.1 ml of the resuspended culture solution to a cuvette and running it in the analyzer. The instrument makes use of the trypan blue exclusion assay in which only dead cells take up the dye and the living cells do not. The same idea was used when using the hemocytometer to count number of live cells. Hemocytometer counts were performed for cell populations that had a concentration <10,000 cells/ml. A 5-fold dilution was made for the hemocytometer count and all 8 quadrants were counted and average number of cells/quad were taken.

3. Drug Preparation. Tamoxifen stock solution (1 mg/ml) was prepared by dissolving 10 mg of Tamoxifen citrate (Sigma Aldrich, St. Louis, MO) in 0.1 ml Dimethylsulfoxide (DMSO) and 10 ml PBS. Control stock solution was prepared by adding 0.1 ml of DMSO in 10 ml of PBS. Both stock solutions were preserved frozen.

4. 4T1 Cell Viability Assays. Cell viability in 4T1 cell populations was studied using cell cloning and cell proliferation assays. Cell cloning studies investigated the ability of a single 4T1 cell to generate clones with the same genetic properties. Cell proliferation studies were used to measure the viability and growth kinetics of the 4T1 cells. For the cloning study, there were control and 5 different treatment groups based on Tamoxifen treatment levels (1, 2, 3, 5, and 7.5 µg/ml) in duplicates. A total of 3000 4T1 cells in 2 ml of DME-10 were seeded in each 16 mm petri plate. The plates were incubated at 37°C with 7.5% CO_2 and colony count was performed after 48 hours under light microscope. The cell proliferation study had control and 2 different Tamoxifen treatment levels (5, and 7.5 µg/ml) with 200,000 4T1 cells in 2 ml DME-10

in each well of a 6-well plate. The cell count and viability were determined every 24 hours for 5 days (120 hours) using the trypan blue exclusion assay in a Vi-Cell cell viability analyzer.

5. Gene Expression Study. 4T1 Cells were harvested from control and five different Tamoxifen treatment groups in duplicates (1, 2, 5, 7.5, and 10 µg/ml), which had an initial population 150,000 cells in 1.1 ml culture solution in each well of a 12-well plate. Harvested cells were analyzed for expression levels of the GAPDH (control), ATR, ATM and TP53 genes. RNA was extracted using PureLink RNA Extraction Kit (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Verso 2-Step qPCR Kit with SYBR Green (Thermo Fisher Scientific, Waltham, MA). Gene expression levels were studied using qPCR with Verso 2-Step QPCR Kit with SYBR Green (Thermo Fisher Scientific, Waltham, MA) and in Bio-Rad iQ5 Multi-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). qPCR was performed using gene-specific primers obtained as Solaris qPCR Gene Expression Assay with the following sequences – GAPDH forward, 5′-GGCTGGCATTGCTCTCAA-3′, reverse, 3′-GCTGTAGCCGTATTCATTGTC-5′, ATR forward, 5′-AGTCACGACTTGCTGAACTG-3′, reverse, 3′-TGAACGTCACCCTTGGA-5′, ATM forward, 5′-CAGGTCTTCCAGATGTGCAAT-3′, reverse, 5′-ACCGCTTCGCTGAGAAAG-3′, TP53 forward, 5′-TACCAGGGCAACTATGGCTT-3′, reverse, 5′-CTGGCAGAATAGCTTATTGAG-3′ (Dharmacon, Thermo Fisher Scientific, Lafayette, CO).

6. RNA Extraction. *RNA* extraction was performed according to manufacturer's specifications. Cell solutions were spun at 2000 X g for 5 minutes and supernatants were removed. Freshly prepared Lysis buffer solution (lysis buffer solution + 1% 2-mercaptoethanol) was added (0.3 ml per sample) to the pellets and spun at 2600 X g for 5 minutes and supernatants were transferred to clean 1.5 ml tubes. Equal volumes of 70% ethanol were added to samples and the mixtures were thoroughly vortexed to remove any visible precipitate. The solutions were transferred to spin cartridges (700 µl at a time

for each sample) and spun at 12000 X g for 15 seconds at room temperature. Flow through was discarded and 700 µl (per sample) of Wash Buffer I was added to spin cartridges and tubes were spun down at 12000 X g for 15 seconds. Flow through was discarded and spin cartridges were added to new tubes. Spin cartridges were washed with 500 µl (per sample) of Wash Buffer II twice and flow through was discarded. Tubes were spun at 12000 X g for 2 minutes to dry the membranes and cartridges were inserted into clean recovery tubes. RNAs were eluted in solution by adding 50 µl (per sample) of RNase-free water to cartridges that were incubated for 1 minute at room temperature and spun at 13000 X g for 2 minutes. Elutes were stored at -20°C and used for cDNA synthesis.

7. cDNA Synthesis. cDNA synthesis was performed in PTC-150 Minicycler™ (MJ Research Inc., Waltham, MA) using a 20 µl reaction mix, consisting of 4 µl 5X cDNA synthesis buffer (1X final concentration), 2 µl dNTP mix (500 µM final concentration for each), 1 µl RNA primers (3:1 random hexamers to anchored oligo-dT), 1 µl RT enhancer, 1 µl Verso™ enzyme mix, 5 µl template RNA and 6 µl DEPC water. The reverse transcription was performed as follows – (i) 1 cycle of cDNA synthesis at 42°C for 30 minutes and (ii) 1 cycle of inactivation at 95°C for 2 minutes. For NEC (No Enzyme Control) reaction, Verso™ Enzyme mix was replaced by 1 µl DEPC water and for NTC (No Template Control) reaction, template RNA was replaced by 5 µl DEPC water.

8. qPCR Analysis. The qPCR reaction mix (25 µl/well) for the 4T1 gene expression study consisted of 12.5 µl 2-step qPCR SYBR Green mix, 1.75 µl gene-specific (GAPDH/ATR/ATM) forward primer (70 nM final concentration), 1.75 µl gene-specific reverse primer (70 nM final concentration), 1 µl cDNA template and 8 µl DEPC water. The thermal cycler was programmed as follows for the qPCR – 1 cycle of initial activation at 95°C for 15 minutes, 40 cycles of (i) denaturation at 95°C for 15 seconds, (ii) annealing at 60°C for 30 seconds and (iii) extension at 72°C for 30 seconds. Melt curve data was obtained using the following program – 81

cycles of melting step at 55°C for 10 seconds, with 0.5°C increments per cycle and 1 cycle of denaturation at 95°C for 30 seconds. The qPCR reaction mix for the gene silencing and toxicity studies used the same reaction mix as above except the following changes –total primer volume was increased to 7.5 µl and volume of DEPC water added was lowered to 4 µl.

9. siRNA Preparation and Storage. Stock concentrations (20 µM) of ATR and ATM siRNAs were prepared by suspending 20 nmols of ATR siRNA dried pellet (Dharmacon, Lafayette, CO) in 1 ml 1X RNase-free siRNA buffer (20 mM KCl, 6mM HEPES-pH 7.5 and 0.2 mM $MgCl_2$) and placing solutions on an orbital shaker for 30 minutes at 0.04 X g in room temperature and centrifuging at 12000 X g for 5 seconds. RNase-free buffer (1X) was prepared by dissolving 200 µl of 5X siRNA buffer (Dharmacon, Lafayette, CO) in 800 µl of RNase free water. Aliquots in 1.5 ml tubes were stored at -20°C.

10. siRNA Transfection. Working solutions (5 µM) of the siRNAs were created by diluting 25 µl of 20 µM solution in 75 µl 1X siRNA buffer. For respective siRNA cocktail (per well), 5 µl of siRNA was added to 95 µl DME-only (no FCS, no Antibiotics) and 1.6 µl DharmaFECT transfection reagent (Dharmacon, Lafayette, CO) was added to 98.4 µl DME-only in separate tubes and incubated for 5 minutes at room temperature to give a final siRNA concentration of 25 nM. The contents of the two tubes were then added together and incubated at room temperature for 20 minutes and then added to respective wells containing 24-hour old culture of 150,000 cells in 800 µl DME-10 (no Antibiotics) each. Transfection cocktails were added to cells seeded 24 hours prior to treatment, after removing the media from wells. The same transfection method was followed in the gene silencing assay followed by clonability.

11. Gene Silencing and Toxicity Assay. Gene-specific silencing was performed in 4T1 cells for ATR, ATM and both using specific, custom designed small interference RNAs (siRNAs)

(Dharmacon, Thermo-Fisher Scientific, Lafayette, CO). Twenty-four hours post-transfection with respective siRNAs, the toxicity assay was performed in two different methods – 1) cell proliferation and 2) cell cloning. In the cell growth kinetics study, 4T1 cells were divided in four categories with approximately 150,000 cells/group based on siRNA treatment – No siRNA (control), ATR, ATM and ATR+ATM (combo). These four categories were subjected to control and two levels of Tamoxifen treatment – 7.5, and 10 µg/ml. Cells were seeded in wells 24 hours prior to siRNA transfection. After 48 hours of Tamoxifen treatment, cells were harvested and counted using a trypan blue exclusion viability assay in a Vi-cell cell viability analyzer and hemocytometer.

For the clonability study, 4T1 cells were divided into four categories of siRNA treatment with 3000 cells/group in duplicates – No siRNA (Control), ATR, ATM and ATR+ATM (Combo). These groups were further subjected to control and two levels of Tamoxifen treatment – 5 and 7.5 µg/ml. Cells were seeded in 16 mm petri plates 24 hours prior to siRNA transfection and Tamoxifen treatment. Forty-eight hours post-Tamoxifen treatment, numbers of colonies were counted under a light microscope. Gene silencing in both studies was confirmed by qPCR analysis for ATR, ATM and GAPDH. Primers were obtained from Integrated DNA Technologies (Coralville, IA) with the following sequences - GAPDH forward, 5′-GGCTGGCATTGCTCTCAA-3′, reverse, 3′-GCTGTAGCCGTATTCATTGTC-5′, ATR forward, 5′-AGTCACGACTTGCTGAACTG-3′, reverse, 3′-TGAACGTCACCCTTGGA-5′, ATM forward, 5′-CAGGTCTTCCAGATGTGCAAT-3′, reverse, 5′-ACCGCTTCGCTGAGAAAG-3′. Primers were resuspended in appropriate volumes of DEPC water – the volume required for each primer was calculated by the following equation:

$$Volume\ of\ DEPC\ water\ for\ resuspension\ (\mu l) = \frac{30,000}{Amount\ of\ Oligo\ at\ OD_{260}\ X\ 33}$$

The amount of oligo at OD_{260} was provided by the manufacturer. Resuspended solutions of forward and reverse primers for the same gene were added together and made up to 1 ml stock solutions which were stored at -20°C.

12. Inhibitor preparation. Stock solution (20 mM) for the caspase-9 inhibitor, z-LEHD-FMK (R & D Systems, Minneapolis, MN), was made by dissolving 1 mg in 62 µl of 99.5% DMSO, and was stored at -20°C. Prior to adding to cell culture, the 100 µM solution of z-LEHD-FMK was prepared in DME-10. The p38 inhibitor, SB203580 (Calbiochem, EMD Chemicals, NJ), was resuspended in 1 ml 99.5% DMSO to create a stock solution of 1 mg/ml and 2 µM SB203580 was added to each well from the stock solution, which was stored at -20°C.

13. Inhibitor Assay. In a 96-well plate, 1 X 10^4 cells in 200 µl DME-10 were plated in four major groups as determined by inhibitor treatment – no inhibitor (control), caspase-9 inhibitor (Z-LEHD-FMK, R&D Systems) p38 inhibitor (SB203580, Calbiochem, (EMD Chemicals, NJ), caspase-9 + p38 inhibitors (Combo). The caspase-9 inhibitor and combo groups received 100 µM final concentration of the inhibitor and the p38 inhibitor and combo groups received a final concentration of 2 µM of p38 inhibitor. These groups were then further divided into four other groups based on Tamoxifen treatment – 0 (control), 5, 7.5 and 10 µg/ml. After 48 hours of Tamoxifen treatment, cells were harvested and counted using a trypan blue exclusion viability assay in a Vi-cell cell viability analyzer and hemocytometer.

14. Statistical Analysis. Data obtained from clonability assay and cell growth kinetics assays were analyzed using one-way ANOVA (Analysis of Variance) in MS Excel 2007. Multiple comparison tests between groups were performed using one-tailed Student's t-Test in MS Excel 2007. Results from gene silencing and toxicity assays were analyzed using a two-way ANOVA in PASW17 software and Microsoft Excel 2007.

RESULTS

Tamoxifen reduces cell viability in a dose-dependent manner. Tamoxifen was seen to decrease cell viability in 4T1 cells in a dose-dependent manner in both the cloning and cell proliferation studies (Figures 1a & b), which is consistent with previous findings by Goel et al (2008). The effect of Tamoxifen is shown to be present in different threshold levels, for example, there is a significant drop in the number of colonies at low concentrations (1 and 2 µg/ml) compared to no Tamoxifen treatment ($p < 0.001$, $\alpha = 0.05$); but there is no significant difference when Tamoxifen concentration is increased from 2 µg/ml to 4 µg/ml ($p = 0.48$, $\alpha = 0.05$) (Figure 1a). An increase in the Tamoxifen concentration from 4 µg/ml to 5 µg/ml crosses a threshold and significantly decreases the number of viable colonies ($p = 0.0003$, $\alpha = 0.05$) – a phenomenon also observed when concentration was increased to 7.5 µg/ml from 5 µg/ml ($p < 0.001$, $\alpha =$

0.05) (Figure 1a). Analysis of data obtained from cell proliferation experiments showed a marked difference ($p = 0.003$, $\alpha = 0.05$) among the two treatment groups and the differences were sustained over 72 hours (Figure 1b). Figure 1b shows the differences between the cell populations in the two different Tamoxifen treatment groups (5 and 7.5 µg/ml) at the peak of their respective growth curves (data not shown) at 72 hours. Although all groups started with the same number of cells, there was a significantly lower number of cells in both the 5 µg/ml ($p = 0.04$, $\alpha = 0.05$) and 7.5 µg/ml ($p = 0.01$, $\alpha = 0.05$) treatment groups. The increase in Tamoxifen concentration from 5 µg/ml to 7.5 µg/ml lead to a significant decrease ($p = 0.002$, $\alpha = 0.05$) in the number of cells, as was seen in the cloning study, thus confirming the dose-dependent action of Tamoxifen.
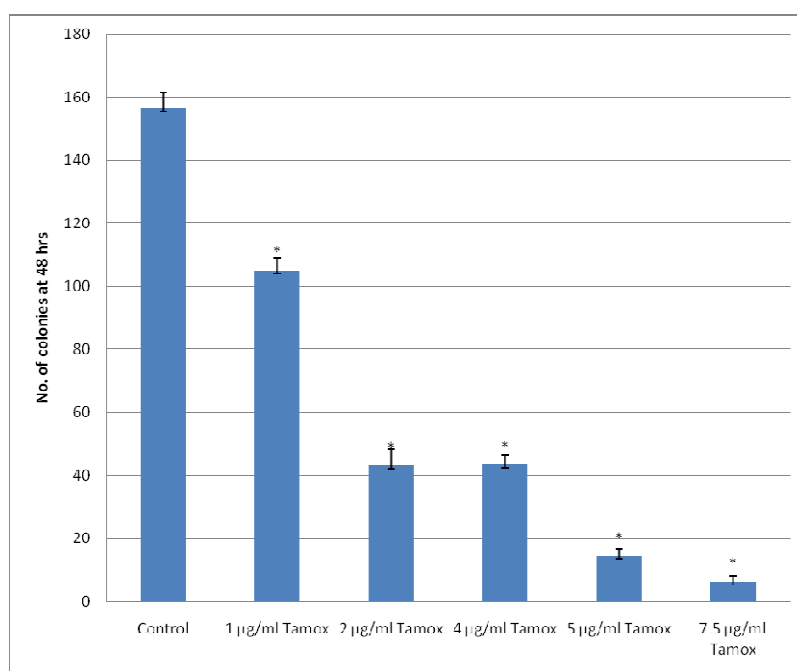

Figure 1a. *Combined clonability data showing number of colonies at 48 hours post-Tamoxifen treatment in different groups. Data from three experiments were pooled and the mean counts are presented with error bars representing standard deviations within observations for each group. (*) represents statistically significant decrease in number of colonies when compared to the Control group.*
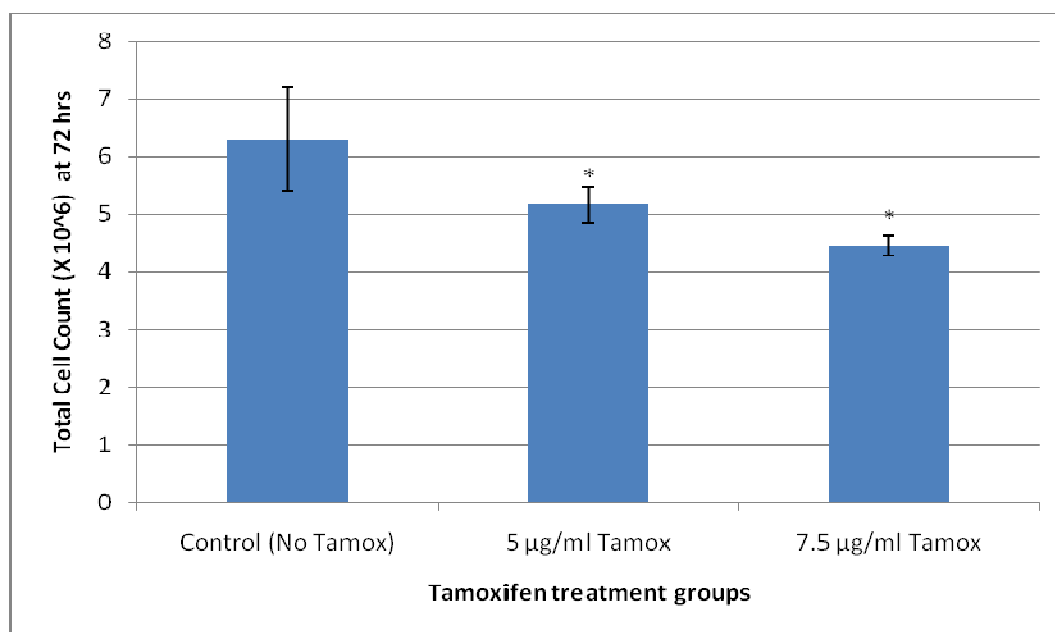
Figure 1b. *Total cell count (X10^6) at 72 hours from the two different Tamoxifen (5 and 7.5 µg/ml) treatment groups. The average of four replicates are shown with error bars representing standard deviation within groups. (*) represents statistically significant decrease in total cell count when compared to the Control (No Tamox) group.*

Tamoxifen induces changes in ATR, ATM and TP53 expression levels. When treated with different levels of Tamoxifen, the gene expression levels for ATR, ATM and TP53 did not vary in a dose-dependent manner, as shown in figure 2. The levels of ATR and ATM hover around an approximate two-fold increase in expression with increasing levels of Tamoxifen treatment compared to no treatment. But when the Tamoxifen concentration was increased from 7.5 µg/ml to 10 µg/ml, the level of expression for ATR decreased below the normal expression level and the ATM expression level decreased down to the basal level, indicating a shift in the mechanism of death from apoptosis to necrosis, which is supported by the expression levels of TP53. The same pattern as with ATR and ATM, is

seen in the TP53 expression level until the 5 µg/ml concentration, i.e., only a two-fold increase is observed in the expression level compared to basal level. When the Tamoxifen concentration is increased to 7.5 µg/ml, TP53 expression is seen to increase approximately 8-fold compared to basal expression level, indicating a high number of cells undergoing apoptosis. When the concentration of Tamoxifen is increased from 7.5 µg/ml to 10 µg/ml, TP53 expression is seen to come down to approximately basal level, supporting the notion that a higher percentage of the cell population are undergoing necrosis rather than apoptosis due to high toxicity of Tamoxifen.
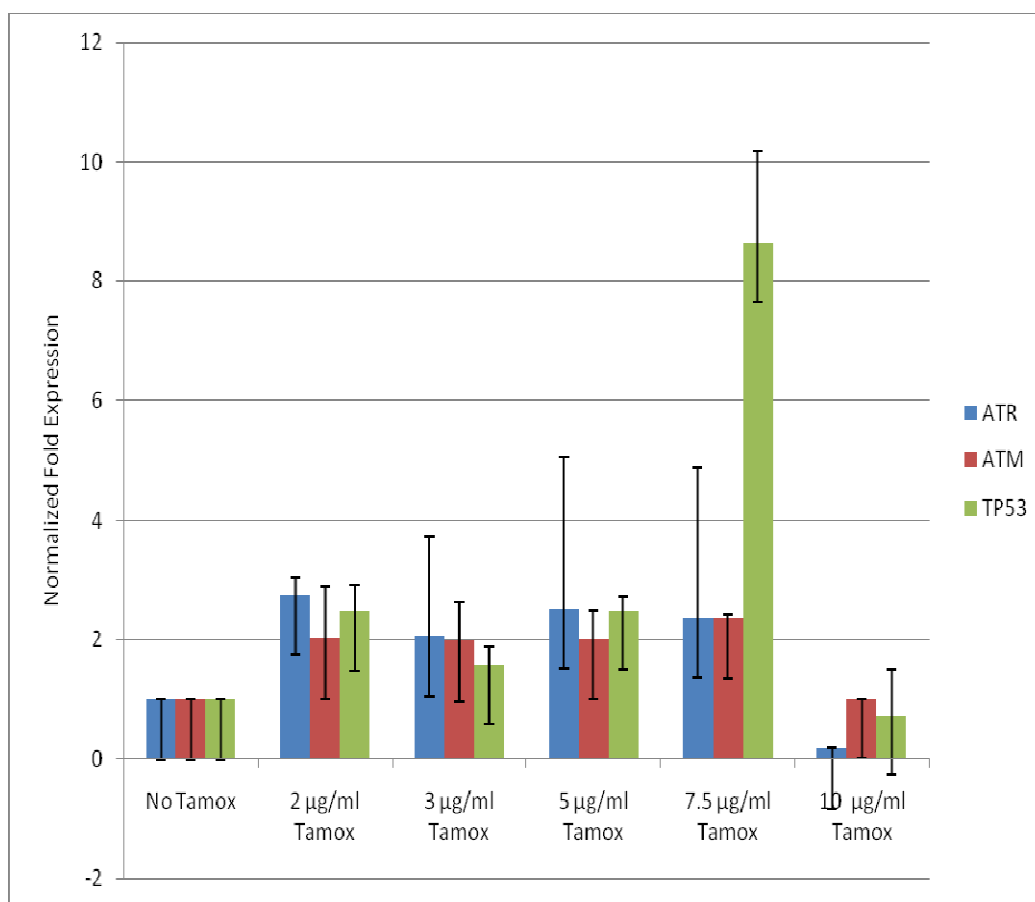
Figure 2. *Gene Expression levels for ATR, ATM and TP53 in cell populations at 48 hours after varying Tamoxifen treatments. Levels are normalized against GAPDH expression levels in all cell populations for basal expression level. Data shown represents mean expression levels from three repeat experiments with error bars representing standard deviation within groups.*

ATR and ATM siRNAs decrease respective gene expression levels. The data from gene silencing using siRNA inhibition (Figure 3a) show that individual siRNA treatments resulted in ~20% inhibition for ATR and ~50% for ATM; and the combination treatment led to ~40% inhibition in both the genes. Because the combination treatment was consistent in decreasing expression of both ATR and ATM, this treatment was used to compare the effects of silencing ATR and ATM on cell viability in response to Tamoxifen treatments (figure 3b). Figure 3b shows that the combination (ATR + ATM) siRNA treatment decreases expressions of ATR and ATM by ~6-fold and ~4-fold, respectively, in presence of 7.5 µg/ml Tamoxifen.
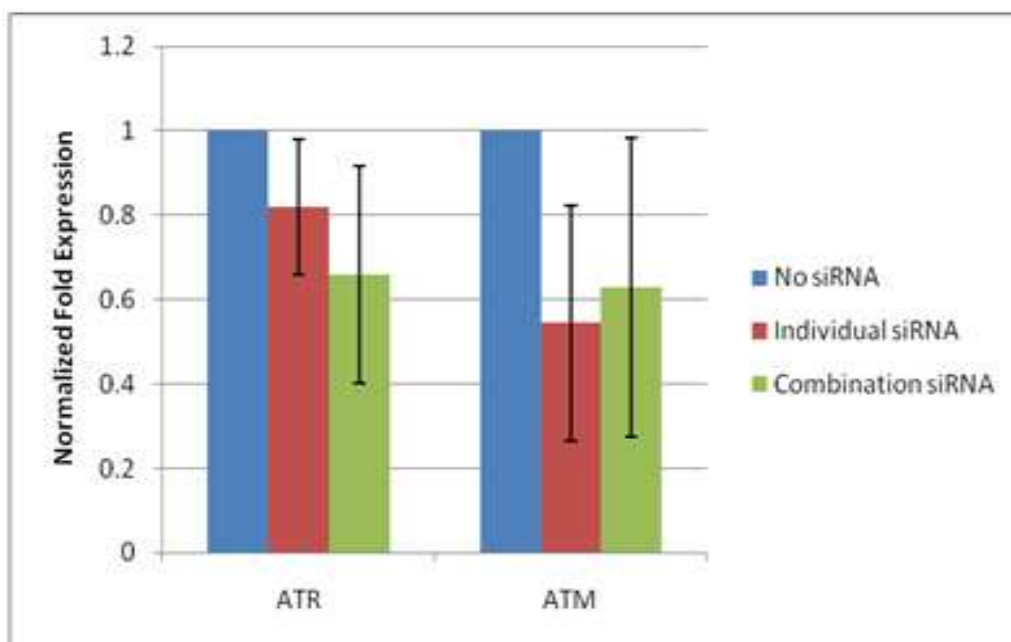
Figure 3a. *ATR and ATM gene inhibition using individual (ATR/ATM) and combination (ATR + ATM) of ATR and ATM-specific siRNAs. Data shows average fold expressions of ATR and ATM normalized to GAPDH expression levels, at different siRNA treatment levels at 48 hours, from three repeated experiments. Error bars represent standard error of the mean (SEM).*
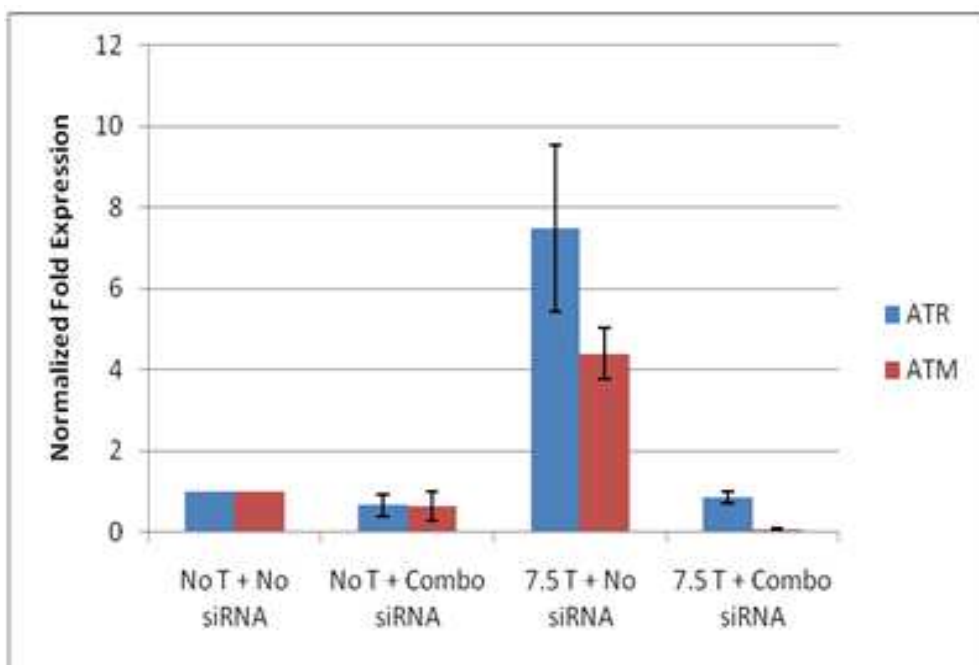

Figure 3b. *ATR and ATM gene inhibition using combination of ATR and ATM-specific siRNAs. Data shows average fold expressions of ATR and ATM normalized to GAPDH expression levels, at control and 7.5 μg/ml Tamoxifen treatment level at 48 hours, from three repeated experiments. Error bars represent standard error of the mean (SEM).*

ATR and ATM gene silencing does not increase cell viability in response to Tamoxifen. The data from the cell cloning experiment done using three different Tamoxifen concentrations following the inhibition of the ATR and ATM genes showed that the silencing of these genes did not significantly affect the response to varying Tamoxifen treatments. As seen in figure 4a, the tamoxifen control (no tamoxifen) group showed significantly higher number of colonies compared to the 7.5 µg/ml treatment groups (p< 0.001, α= 0.05), regardless of siRNA treatment. In contrast, siRNA treatment did not significantly affect colony formation within the same Tamoxifen treatment regimen (p= 0.186, α= 0.05), even after inhibiting ATR by ~6-fold and ATM by ~4-fold in the 7.5 µg/ml treatment group (figure 3b). In the Tamoxifen control groups, a 40% inhibition of ATR and ATM (figure 3b) did not significantly affect the number of colonies formed (figure 4a). The cell proliferation studies with siRNA and Tamoxifen treatments showed a similar trend – the 7.5 µg/ml Tamoxifen treatment groups have significantly lower total cell counts (p< 0.001, α= 0.05) compared to the control (no Tamoxifen) treatment groups (Figure 4b), regardless of inhibition of ATR and ATM genes.
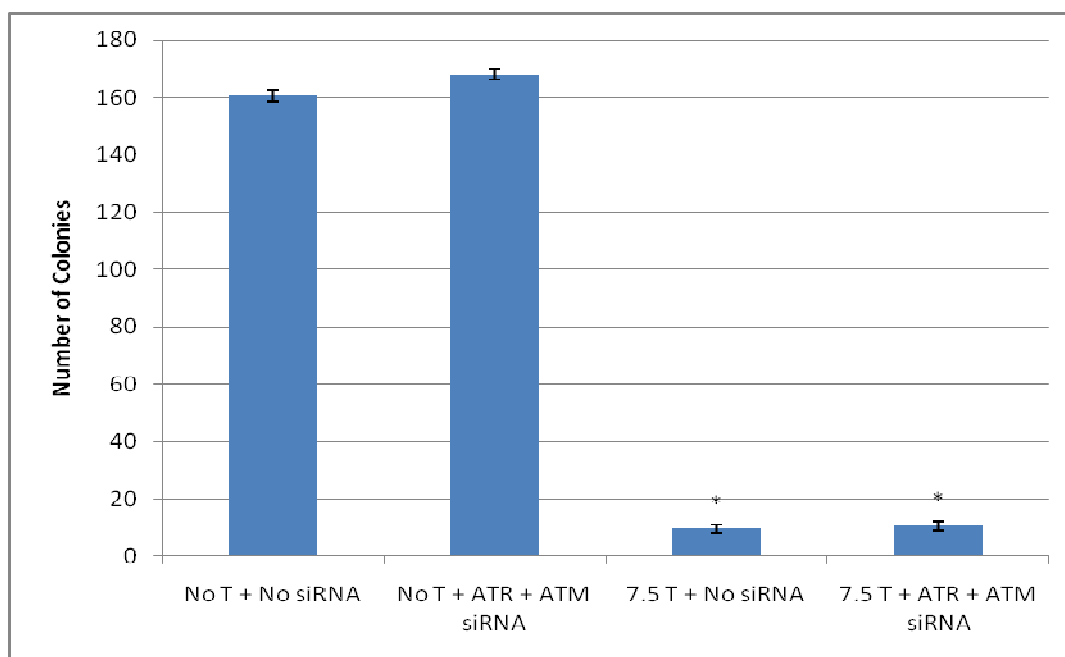

Figure 4a. *Total number of colonies formed at 48 hours after Tamoxifen and siRNA treatments. Data shown represents average number of colonies from 3 repeat experiments, each with two replicates; error bars represent standard deviation among data points. (*) represents statistically significant decrease in number of colonies when compared to the control group (no Tamoxifen + no siRNA).*
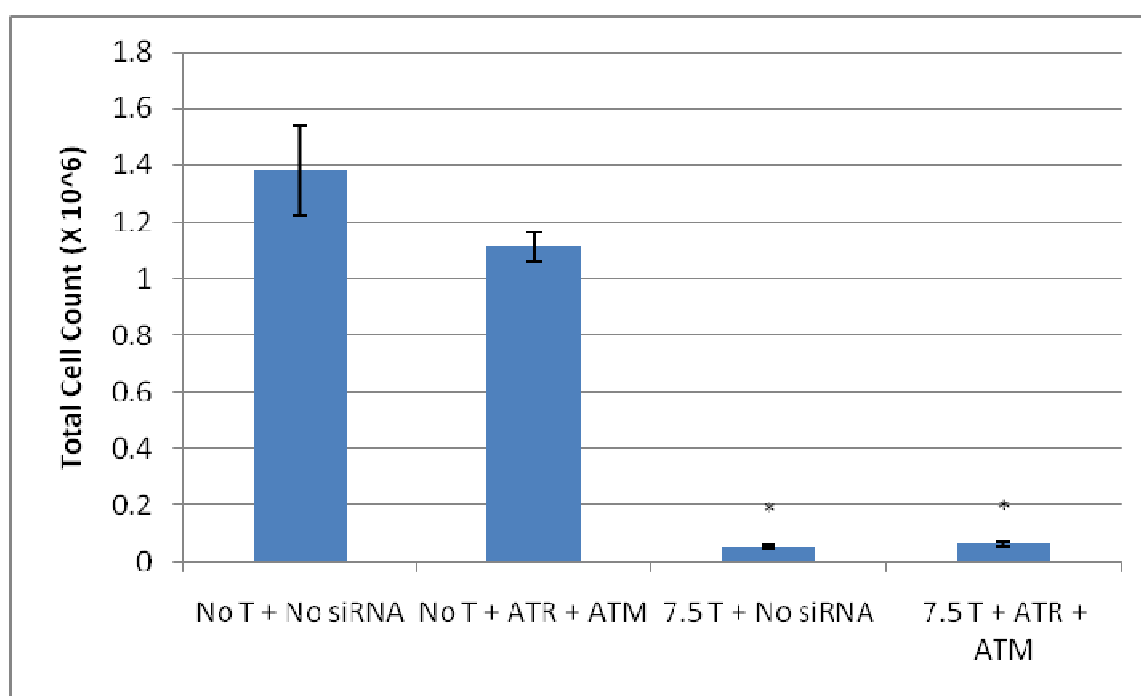
Figure 4b. *Total cell count (X10^6) at 48 hours after Tamoxifen and siRNA treatments. Data shown represents average total number of cells from 3 repeat experiments, each with two replicates; error bars represent standard deviation among data points. (*) represents statistically significant decrease in number of colonies when compared to the control group (no Tamoxifen + no siRNA).*

Inhibition of caspase-9 significantly increases cell viability in response to Tamoxifen. The possibility that the ATR-ATM-TP53 pathway does not play a significant role in Tamoxifen-induced apoptosis was reinforced by the data obtained from the inhibitor study, as shown in figure 5. Treatment with caspase-9 inhibitor brings about significant increase in viability when compared to the no inhibitor group at both 5 and 7.5 µg/ml Tamoxifen treatment levels (p= 0.0001 at 5 µg/ml, p< 0.001 at 7.5 µg/ml, α= 0.05). The treatment with p38 inhibitor did not increase cell viability; instead, it decreased cell viability significantly when

Tamoxifen was not present (p< 0.001, α= 0.05). When treated with both p38 and caspase-9 inhibitors, cellular viability increased compared to no inhibitor group at the 5 and 7.5 µg/ml Tamoxifen treatment levels (p= 0.0039 for 5 µg/ml, p= 0.0016 for 7.5 µg/ml, α= 0.05). This shows that caspase-9 inhibition was the most effective in increasing cell viability when treated with Tamoxifen. This finding suggests that caspase-mediated apoptosis was probably the most significant ER-independent pathway for Tamoxifen-induced apoptosis.
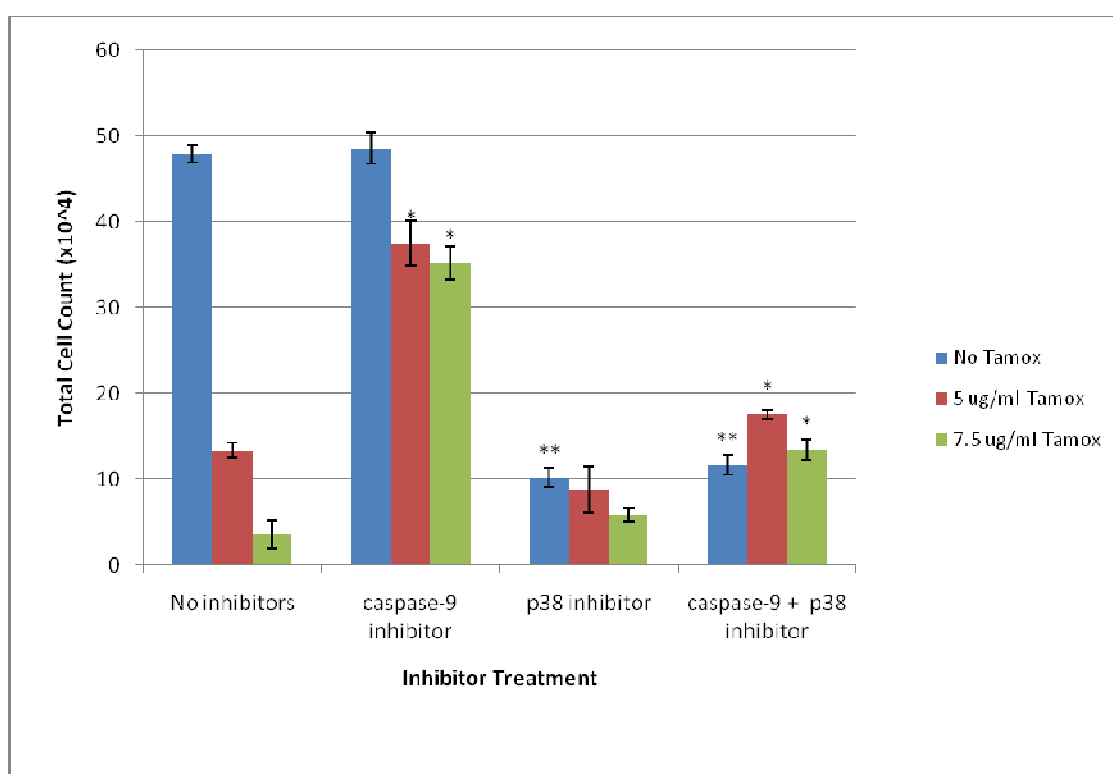
Figure 5. *Total cell count of populations at 48 hours following varying Tamoxifen and inhibitor treatments. Data shown are average of 2 repeat experiments with 2 replicates each; error bars represent standard deviation of data points. (*) represents statistically significant increase in cell count compared to no inhibitor treatment groups at the same Tamoxifen treatment levels (5 µg/ml and 7.5 µg/ml). (**) represents statistically significant decrease in cell count compared to the no inhibitor treatment group at the no tamoxifen treatment level.*

DISCUSSION

The results obtained from this research show the following trends – 1) Tamoxifen decreases cell viability and growth in a dose-dependent manner (figures 1a & b) and 2) Tamoxifen increases levels of expression of the ATR, ATM and the TP53 (also called p53) genes (figure 2 & 3b), 3) inhibiting the expression of both ATR and ATM genes do not seem to have a significant effect on the viability of the cells when treated with the apoptosis-inducing concentration (7.5 µg/ml) of Tamoxifen (figures 3a, b, 4a, b & 5).

The first trend has been observed in multiple previous studies done with Tamoxifen (Obrero et al 2002; Goel et al 2008) and confirms the action of Tamoxifen on ER(+) breast cancer cells. The second

trend was seen in both the gene expression and the gene silencing studies. As seen in figure 2, treatment with Tamoxifen doubles ATR and ATM gene expression until 10 µg/ml Tamoxifen. The same pattern is seen for TP53 expression until the 7.5 µg/ml treatment. The increase in the level of TP53, especially at the 7.5 µg/ml Tamoxifen treatment level, is consistent with the findings that high expression of TP53 is usually related to increased apoptosis in cell populations (Ayala et al 2007; Böttger et al 2008). The stability of the TP53 expression levels at lower concentrations of Tamoxifen (figure 2) can be explained by the finding that TP53 is able to initiate apoptosis through transcription-independent pathways such as through interactions with members of the Bcl-2 family proteins (Speidel 2009). But it is to be noted that the TP53 expression at 7.5 µg/ml Tamoxifen

treatment increases by ~7-fold compared to the 2-fold of the lower Tamoxifen concentrations. Since Tamoxifen did not increase the expression of TP53 in a dose-dependent manner (figure 2), the relation between expression levels of TP53 and its role in Tamoxifen-induced apoptosis is not clear – a phenomenon that has been observed previously in the literature (Renoir et al 2008). The study by Tao et al (2008) show the presence of the TP53 protein product whereas Sang et al (2005) had shown the 4T1 cell line to have a p53 null mutation, thus causing a dispute over the presence of p53 protein in 4T1 cells. One study reported decreased TP53 expression with decrease in Tamoxifen concentration (Dinda et al 2002), and that Tamoxifen induces transcription of TP53 through the P1 promoter in MCF-7 cells (ER(+)) (Hurd et al 1997). Studies have also reported no change in TP53 levels with high levels of apoptosis in cell populations as indicated by high pRb dephosphorylation (Fattman et al 1998; Zhang et al 1999). The reason behind such conflicting data was suggested to be the different subcellular TP53 distribution within cells (Renoir et al 2008). The high TP53 expression at the 7.5 µg/ml concentration (figure 2) could be explained by the finding that TP53 is responsible for the induction of ~150 apoptosis-related genes encoding several protein families, which requires increased levels of TP53 expression (Fridman and Lowe 2003). Also, the ER-independent action of Tamoxifen, which mainly induces apoptosis through the mitochondrial caspase pathway, requires high expression of TP53 (Mandlekar and Kong 2001; Obrero et al 2002). This is supported by the finding that the caspase inhibitor z-VAD-fmk completely blocks Tamoxifen-induced apoptosis in ER(+) cells (Mandlekar et al 2000; Renoir et al 2008). Moreover, it has been suggested that in ER(+) cells, the Tamoxifen-induced reactive oxidative species (ROS) production responsible for DNA damage (Wozniak et al 2007), is associated with release of mitochondrial cytochrome c which mediates apoptosis through the caspase pathways and extracellular signal-regulated kinase signaling, all of which involves p53 protein (Renoir et al 2008; Zheng et al 2007). In contrast, studies show that for

classical caspase-mediated apoptosis, p53 plays a key role in activating the caspase cascade without an increase in p53 expression (Schuler and Green 2001). In the same study, it was seen that p53 can non-transcriptionally induce expression of Bcl-2, which is an intermediate signaling molecule between the caspases and p53 (Schuler and Green 2001). Thus, it can be hypothesized that in case of Tamoxifen-induced apoptosis, transcriptional induction of p53 is not required for caspase activation. Also, Tamoxifen-induced ROS production causes mitochondrial dysfunction, which results in caspase activation through a p53-independent pathway such as the Rb-E2f pathway (Polager and Ginsberg 2009).

The third trend observed can be explained in many ways. First of all, ATR and ATM proteins are detection proteins situated at the top of a cascade of proteins which involve a multitude of proteins and cofactors (Sengupta and Harris 2005; Morgan 2007). Thus, inhibition of ATR and ATM proteins will not necessarily mean a decrease in TP53 function since TP53 exerts its action in many other pathways. Another significant reason is that because 4T1 cells are ER(+), it is possible that at the 7.5 µg/ml concentration, Tamoxifen will exert its actions on 4T1 cells through the ER-dependent pathway rather than ER-independent pathways which include the ATR-ATM-TP53 pathway. And, among the ER-independent pathways, the caspase-mediated mechanism of apoptosis is the dominant one as shown by previous findings in which caspase inhibition completely restored cell viability (Mandlekar et al 2000; Renoir et al 2008). As seen in figure 5, inhibition of caspase-9 significantly increased cellular viability under different Tamoxifen treatments. The purpose of the caspase-9 and p38 inhibitor study was to force the cells to undergo apoptosis through the ATR-ATM-TP53 pathway since the caspase pathway and the p38/JnK pathway were the dominant ER-independent pathways (Mandlekar and Kong 2001). Although the caspase-9 inhibitor worked very well, consistent with the findings of Morishima et al (2008), the results from the p38 inhibitor groups in this study were not consistent with previous findings by

Obrero et al (2002) that showed strong restoration of cell viability by the p38 inhibitor. However, given that SB203580 only inhibits p38 and not JnK (Obrero et al 2002), it is possible that apoptosis was not inhibited due to differential JnK signaling in apoptotic pathways that do not involve activation of p38 (Whitmarsh et al 1997). In contrast, co-activation of both JnK and p38 is required to mediate apoptosis and the failure to do so would actually result in growth of cells through the ERK pathway (Xia et al 1995). Because this pathway is very complex and recently JnK has been shown to actually have both pro and anti apoptotic behavior (Liu and Lin 2005), it should be studied more in depth in 4T1 cells in response to Tamoxifen treatment.

A major source of variation in gene expression data following siRNA-mediated silencing was the inhibition efficiency. Although inhibition was observed with combination treatment, the inhibition levels were not that prominent in the no tamoxifen treatment regimen (figures 3a & b). This can be attributed to the fact that only a single siRNA was used for each gene, whereas multiple siRNAs can be used for the same gene and also different concentrations can be used for the siRNAs. Apart from siRNA concentration or different siRNA sequences, another factor that affected inhibition efficiency was the type of transfection reagent and the concentration used. Preliminary studies (data not shown) done using the HiPerfect Transfection reagent from Qiagen (Valencia, CA) showed a lower inhibition compared to the DharmaFECT transfection reagent used throughout the study. Also various other factors that contribute to siRNA efficiency such as hybridization thermodynamics, accessibility to target site, cell-specific parameters (cell line, degradation, cross-hybridization, etc.), strand asymmetry, etc., could have affected the inhibition levels (Panceska et al 2004; Kurreck 2006). In future, the siRNA concentration and corresponding transfection reagent type and concentration should be optimized for achieving higher levels of inhibition. Even alternative methods can be considered for gene inhibition such as knockouts using Cre-lox system, siRNA delivery through lentivirals, etc., in future studies.

CONCLUSION

The aim of the study was to determine if the proteins ATR and ATM play a role in the apoptosis induced by Tamoxifen. Based on data collected, it can be concluded that ATR and ATM do not play a significant role in bringing about Tamoxifen-induced apoptosis, contrary to the initial hypothesis. It must be noted, however, that this study used an ER(+) breast cancer cell line, and the findings may differ if the same study was performed using ER(-) cells. Future studies can be based on similar investigations into different breast cancer cell lines to elucidate the complete mechanism of Tamoxifen's action. This greater understanding will possibly allow for prevention of the serious side-effects and next-generation drug development.

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