THE EFFECTS OF 17β-ESTRADIOL AND TAMOXIFEN ON CELL CYCLE DISTRIBUTION AND F-ACTIN EXPRESSION IN MCF-7 CELLS

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ABSTRACT

A large body of research has focused on the effects of estrogens and anti-estrogens such as tamoxifen on cancer tumor growth. Estrogen receptors (ER) refer to ligand-dependent transcription factors that regulate maintenance, growth and differentiation of activities within cells, including MCF-7 cells. Tamoxifen (TAM) molecules compete for ER sites, thereby decreasing the effects of estrogen-stimulated breast cancer cell proliferation. However, there is also experimental indication that TAM may cause cancer in long-term treatment in other reproductive tissues. Of similar interest is the influence of 17-β estradiol (E_2) itself, which is purported to have synergistic effects on cell proliferation. In this experiment we have examined the influence of TAM (10^{-6}M and 10^{-4}M) and E_2 (10^{-6}M and 10^{-4}M) on estrogen-responsive MCF-7 cell progression. Flow cytometric analysis was utilized to determine the effect on cell cycle distribution in MCF-7 breast cancer cells upon treatment over 24 hours incubation period. The analysis disclosed that TAM (10^{-4}M) and E_2 and TAM (10^{-4}M) combined treatments affected the G_0/G_1, S and G_2/M phases of MCF-7 cells compared to the controls, and E_2 (10^{-4}M) showed a decrease in the S phase. It was also observed that treatment with 10^{-4}M TAM induced apoptosis. These data suggest that tamoxifen on MCF-7 cells is growth inhibitory, cell cycle perturbing and apoptotic, whereas natural estrogen does not share all of these responses. Localization of F-actin was examined by indirect immunofluorescence microscopy using rhodamine-conjugated phalliodin to label filamentous actin. When compared to control, 10^{-4}M 17β-estradiol and 10^{-4}M tamoxifen exhibited shortened and less microfilament protein in MCF-7 cells. Western blot analysis indicated a marked decrease of F-actin expression in these cells by 10^{-4}M TAM and a moderate decrease by 10^{-4}M E_2. This provides a useful model to study post-translational modification of F-actin regulation of cytoskeleton in ER responsive breast cancer tumor cells.

INTRODUCTION

For years, the effects of tamoxifen were thought to act through a mechanism of blocking estrogen receptors and acting as an anti-estrogen (Kennedy, 1962; Heel et al., 1978; Kayalar et al., 1996; Forbes, 1997). One in three women who experience metastatic mammary cancer responds favorably to treatment with estrogens (Davidson and Lippman, 1995). Subsequent use of surgery as a medical approach to treatment of breast cancer has surpassed the use of anti-estrogens and aromatase inhibitors (Santen et al., 1981). However, high dose estrogen (hormone-additive) therapy also generally results in tumor regression in postmenopausal female patients with breast cancer that is estrogen receptor (ER) positive (Kennedy, 1962). In another study ZR-75-1, an estrogen positive breast cancer cell line was investigated utilizing serum-free medium. This research

indicated that estradiol has proliferative effects at 10^{-8} M and 10^{-9} M, and tamoxifen also has stimulatory effects in a dose-dependent manner up to a concentration of 10^{-7}M (Cover et al., 1999). Here we see the ability of tamoxifen to mimic the action of 17-β estradiol. In a 1993 study, it was determined that under certain physiological conditions (decreasing lipoprotein lipase activity and parametrial adipose white tissue wet weight) tamoxifen (an anti-estrogen) mimicked the action of estradiol (Wade and Heller, 1993). Since tamoxifen mimics and also acts as an anti-estrogen in varying conditions, the current study was undertaken to test the effects of tamoxifen and estradiol on the ERα-positive MCF-7 cell line. Various studies have also shown that the nuclear receptor, ERα mediates the expression of the protooncogene c-fos gene as activated by 17-β estradiol in ERα positive breast cancer cell lines (Wilding et al., 1988; Van der Burg et al., 1989, 1990, 1991; Doucas et al., 1991; Weisz and Bresciani, 1993; Bonapace et al., 1996; Duan et al., 1998).

Tamoxifen is a non-steroidal triphenyl ethylene and it is able to structurally conform in a manner that makes it look like steroids within the nucleus. It is therefore able to bind to the ER receptors, thereby acting as a competitive inhibitor to estradiol (Skidmore et al., 1972; Jordan and Prestwich, 1977). One mechanism in which anti-estrogens such as tamoxifen is believed to function may possibly be by holding the dividing cell in the G1 phase of the cell cycle, thereby decreasing cell proliferation (Sutherland et al., 1983; Osborne et al., 1984). The role of the cell cycle as it pertains to program cell death or apoptosis in response to tamoxifen or other hormonal treatment, such as estrogen, is of our interest to study. In one 72 hour study, it was determined that for MCF-7 cells, mRNA and protein levels associated with ER expression was slightly elevated or unregulated with very low concentrations of 10^{-10}M and 10^{-11}M estradiol whereas higher concentrations reduced mRNA and protein expression over the 72 hour study period (Ree et al., 1989). In this study, we examined the effects of estradiol and its tamoxifen counterpart over a 24-hour incubation period. We tested if there were any effects as early as one day and the nature of these effects on the cell cycle of MCF-7 cells at relatively higher dosages.

Specifically we treated MCF-7 cells *in vitro* with dual concentrations of tamoxifen and 17β-estradiol (10^{-4}M, 10^{-6}M) as well as combined treatments of tamoxifen and 17-β estradiol (10^{-4}M, 10^{-6}M) for a 24-hour incubation period. Flow cytometric analysis was conducted to determine the effects of tamoxifen and 17β-estradiol on cell cycle distribution.

Filamentous actin (F-actin) protein is a major intracellular component of human cytoskeletal structure that is involved in cell motility and adhesion. Moreover, functions of actin filaments include forming a band just beneath the plasma membrane that anchors the centrosomes at opposite poles of the cell during mitosis and the generation of cytoplasmic streaming in some cells. This protein molecule is also responsible for pinching and dividing animal cells apart during cytokinesis. Thus, its regulation is of particular interest as an underlying pathway in estrogen responsive cells when exposed to estrogens or anti-estrogens. Therefore, we have studied the expression of F-actin in MCF-7 cells following treatment with E_2 and TAM. One observed morphological change in apoptosis or PCD is cell condensation and the reorganizing of the cell cytoskeleton (Tenniswood, 1992). Cell preparation and execution of apoptosis is associated with depolymerization and cleavage of actin as well as other cytoskeletal proteins (Bonfocco

et al., 1996; Chen et al., 1996; Kayalar et al., 1996; Levee et al., 1996; Brancholini et al., 1997; Brown et al., 1997; Caulin et al., 1997; Kothakota et al., 1997; Ku et al., 1997; Kruiudering et al., 1998; Porter and Janicke, 1999; Schmeiser and Grand, 1999). For this reason, we used indirect immunofluorescence microscopy and western blotting to test whether treatments of varying concentrations of tamoxifen and 17β-estradiol had any effects on the expression of the cytoskeletal protein, F-actin.

MATERIALS AND METHODS

Cell Culture. MCF-7 human mammary adenocarcinoma cell line was obtained from American Type Culture Collection (Rockville, MD) and routinely maintained in Dulbecco's Modified Eagle's/ F-12 media (DMEM; Sigma Chemical Co., St. Louis, MO) with 10% charcoal dextran-treated fetal bovine serum (FBS). Cells were cultured in a 5% CO_2 incubator at 37°C.

For western blotting, cells (1.0×10^6 cells) were cultured on eight 60 mm plates for 24 hours in 10% charcoal dextran-treated fetal bovine serum (charcoal-stripped FBS) at 37°C in 5% CO_2 and cultured in phenol red-free DMEM for 24 hours. For flow cytometry, 5.0×10^5 cells were plated on eight 35mm plates in duplicate. After 24 hours incubation, media was replaced with 10% charcoal-stripped serum containing media (control), media containing 0.1%DMSO (as vehicle control) or media containing E_2 (10^{-6} M, 10^{-4} M), TAM (10^{-6} M, 10^{-4} M), E_2+TAM (10^{-6} M, 10^{-4} M), respectively.

Flow Cytometry. After 24 hours incubation period, cells were trypsinized (trypsin-EDTA) and centrifuged at 3500 rpms for five minutes. Cell pellet from each treatment group were stained with 1ml Vindelov's Propidium Iodide (PI) solution (10mM Tris-HCl, 10µg/ml RNase, 10 mM NaCl, and 0.1 mg PI/ml and 2.0% NP-40) before analyzing the DNA ploidy using a FACScan flow-cytometer (XL-MCL; Coulter Corporation, Inc., Miami, FL) equipped with a 15mW air-cool laser at 488 nm wavelength. Nuclear DNA content was examined based on the intercalation of PI as red fluorescence.

Fluorescence Microscopy. Cells were grown for 24 hours on square 22 x 22 mm coverglasses in respective treatments. Cells were then washed in phosphate buffered saline (PBS), pH 7.4, and were fixed in 3.0% paraformaldehyde solution for 10 minutes followed by washing twice in PBS containing 0.1% Tween-20. Cells were then incubated for 5 minutes at room temperature with 5% bovine serum albumin in PBS with 0.1% Triton-X-100. Five microliter of a methanolic stock (containing phallodin) was dissolved into 200µl of PBS for each coverslip to be stained. The staining solutions were placed on the coverslip for 20 minutes at RT. During incubation coverslips were kept in a covered container to prevent evaporation. The stained cells were then washed twice with PBS and mounted with Prolong antifade mounting medium (Molecular Probes, Eugene, OR) and viewed under a Zeiss fluorescence microscope.

Western blot Analysis. After the indicated treatments, cell protein was extracted in M-Per (Mammalian protein extraction reagent, Pierce, Rockford, IL). Extracts were centrifuged at 13,000 rpm for 10 minutes. Protein assay was performed on the supernatant using a BCA protein assay kit (Pierce, Rockford, IL). Ten micrograms of protein samples were loaded in each lane, and separated by 10 % SDS-PAGE. Proteins were transferred at 200 V to Hybond-ECL nitrocellulose membranes (Pharmacia Biotech, Buckinghamshire, UK) and blocked for 1 hour at 4˚C in 1% Blotto non-fat dry milk

(Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were incubated with 0.5 μg/ml monoclonal anti-actin (Sigma Chemical Co., St. Louis, MO) for 1 hr at room temperature and subsequently in 1 μg/ml peroxidase conjugated anti-mouse IgG (Fab specific) (Sigma Chemical Co., St. Louis, MO) at room temperature. ECL western blotting detection analysis (Amersham Biosciences, Buckinghamshire, UK) was used to visualize F-actin expression on X-ray film (Eastman Kodak Co., Rochester, NY).

Statistical Analysis. Flow cytometric experiments were repeated seven times with two dishes per treatment and mean and standard error mean (SEM) were calculated. Differences between the various treatment groups were analyzed by one-way repeated measures analysis of variance (ANOVA). The groups that registered significant differences ($p<0.05$) were analyzed by multiple comparison tests. Prism 3.02 software (Graphpad Inc., San Diego, CA) was utilized to graph and analyze flow cytometry data.

RESULTS

When compared with controls, the cell cycle data indicated that 10^{-4}M E_2 significantly ($p<0.05$) downregulated S-phase DNA, while G_0/G_1 and G_2/M DNA remained unaffected. 10^{-4}M TAM and E_2+TAM combined (10^{-4} M), decreased ($p<0.05$) DNA in all phases of cell cycle (Table 1)

Table 1: Effects of E_2, TAM, and E_2+TAM on cell cycle analysis in MCF-7 cells as determined by flow cytometry.

Treatments	Phases of cell cycle		
	G_0/G_1	**S**	**G_2/M**
Charcoal-stripped FBS	64.6±1.6	12.1±1.3	15.3±1.1
0.1% DMSO	62.0±1.3	14.5±1.7	14.7±1.1
E_2 10^{-6}M	62.0±1.2	15.7±1.5	13.4±1.0
E_2 10^{-4}M	68.3±1.7	6.6±0.8 [b]	18.9±1.6
TAM 10^{-6}M	67.5±1.8	12.4±0.6	13.3±1.4
TAM 10^{-4}M	25.8±8.4 [b]	9.8±0.9 [b]	9.6±1.3 [b]
E_2+TAM 10^{-6}M	60.1±1.1	17.3±1.3	13.8±1.3
E_2+TAM 10^{-4}M	19.5±5.8 [b]	6.6±1.5 [b]	3.1±0.7 [b]

Data represents the mean and SEM from 7 separate experiments.

Figure 1 presents results of the representative DNA contents in different phases of cell cycle as determined by flow cytometry. Gated histograms demonstrated peaks at the cell cycle intervals under the treatment regimen except with TAM 10^{-4}M treatment, where a broader peak prior to G_0/G_1 phase indicated apoptosis.

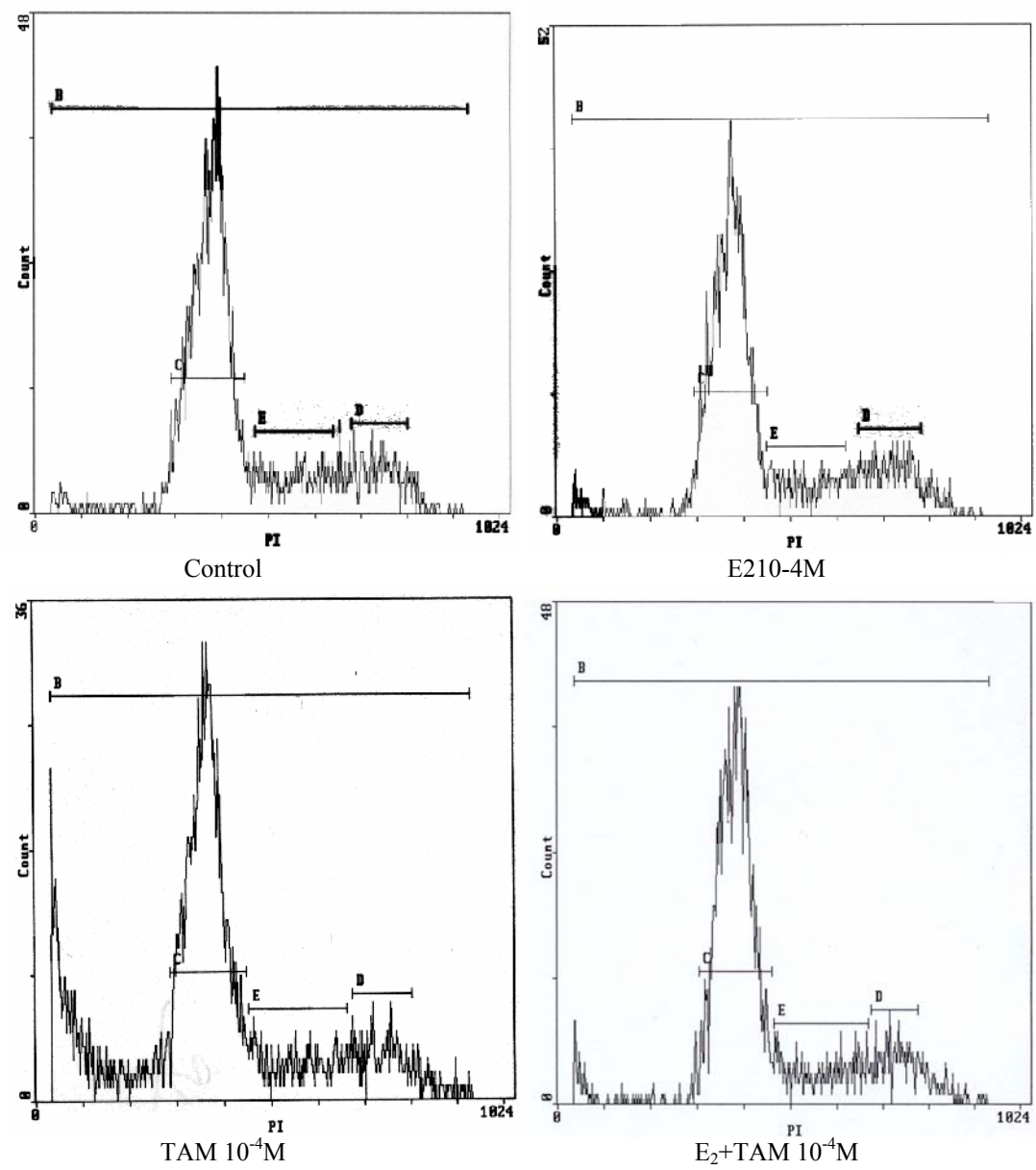

Figure 1: DNA histograms from the representative treatments as determined by flow cytometry.

Photomicrographs presented in Figure 2 showed morphological changes. There was reduced growth and larger vacant spaces on cultures following treatment with 10^{-4}M TAM (D) and 10^{-4}M E_2 (B). Cells treated with 10^{-4}M E_2 (B) registered relatively larger size and rounded shape with more vacant space and cytoplasmic extensions

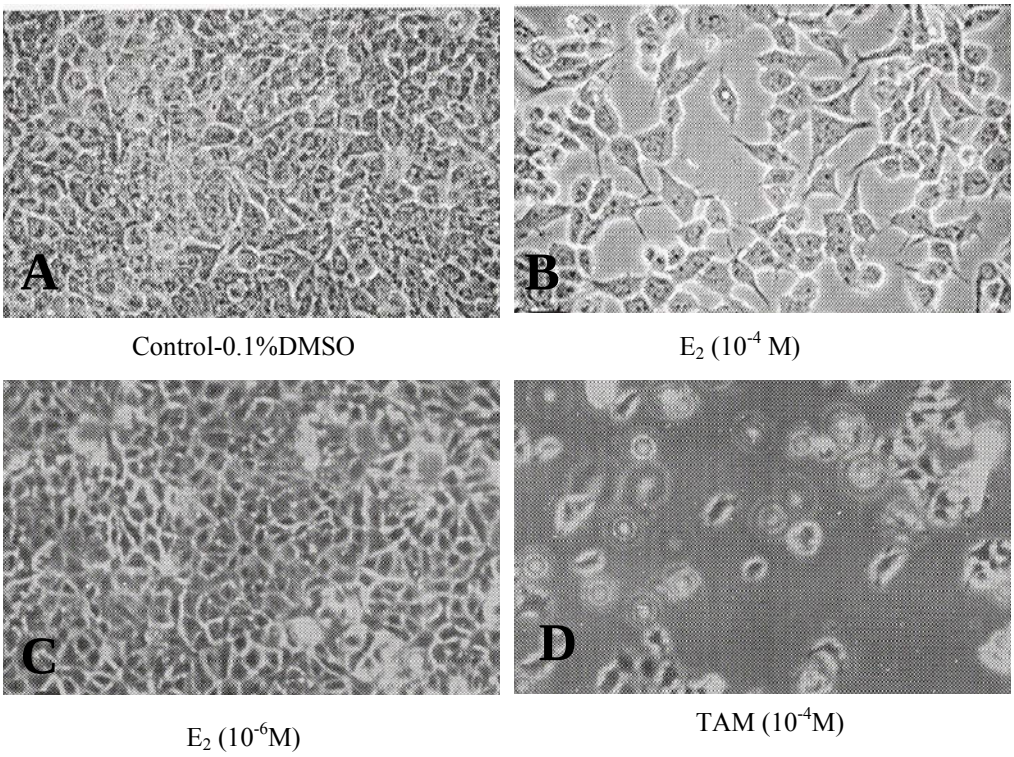

Figure 2. Phase-contrast photomicrographs of cultured MCF-7 cells incubated for 24 hours in medium containing 0.1% DMSO vehicle (A), or in medium with vehicle containing 10-4M E2 (B), 10-6M E2 (C) and 10-4M TAM (D). (270x).

than those in the control cultures (A). There was rounding of and clumping of cells evident in cultures treated with 10^{-4}M TAM (D) and combination of 10^{-4}M E_2+TAM. Detachment of cells from the substrata was observed with higher concentrations of E_2 (10^{-4}M) and more so with TAM (10^{-4} M). Cells treated with10^{-6}M E_2 (C) showed an intense degree of packing and a marked lack of intercellular spaces as compared to even the control cultures.

Localization of F-actin was examined by indirect immunofluorescence microscopy using rhodamine-conjugated phalliodin to label filamentous actin. As demonstrated in Figure 3, 10-4M E2 (B) and 10-4M TAM (D) exhibited shortened and less microfilament. MCF-7 cell cytoskeletal integrity was compromised with both 10-4M E2 and 10-4M TAM, while with 10-6M E2 (C) the cells exhibited well-organized highly intense F-actin staining when compared to that of the control (A).

Western blot analysis indicated a marked decrease to almost none of F-actin expression by 10-4M TAM and a moderate decrease following the 24 hour incubation by 10-4 M E2, As the concentrations of 17β-estradiol and tamoxifen decreased (10-6M E2 and 10-6M TAM) F-actin expression became stronger (Fig. 4).

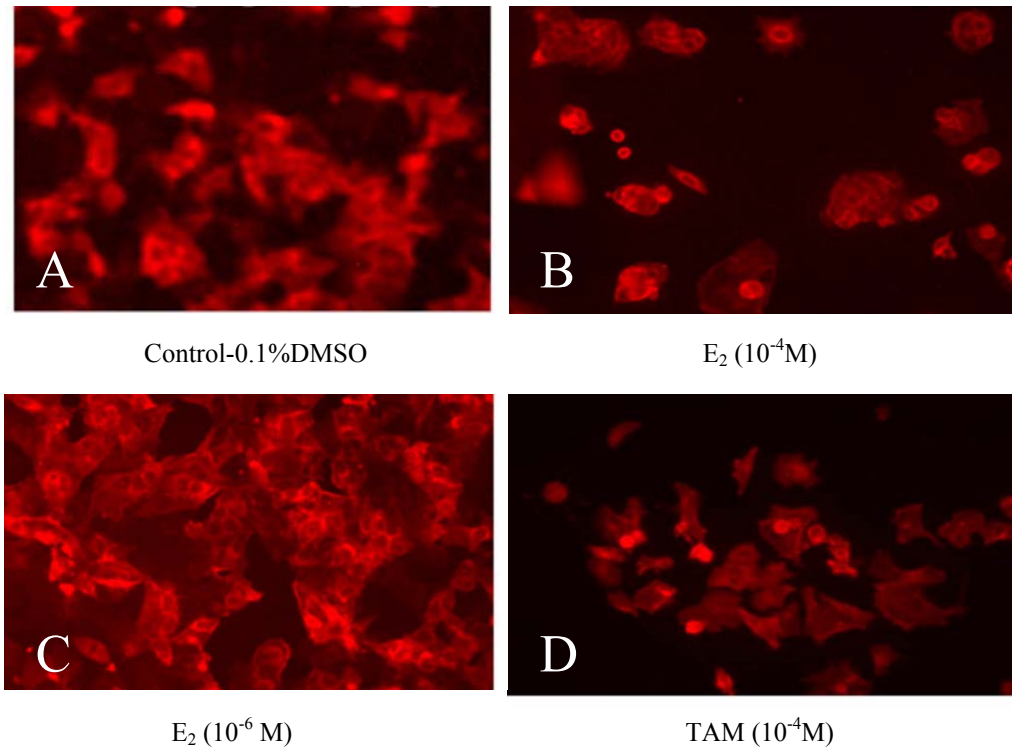

Figure 3. Photomicrographs rhodamine-phalloidin-labeled filamentous actin localization in cultured MCF-7 cells. Cells were cultured on coverslips and incubated for 24 hours in medium containing 0.1% DMSO vehicle (A), or in medium with vehicle containing 10-4M E2 (B), 10-6M E2 (C) and 10-4M TAM (D) (270x).

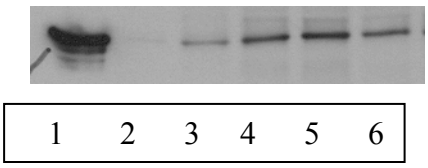

Figure 4: Western blot with 20 µg proteins of rabbit muscle actin as positive control (Lane 1), MCF-7 cell lysates following 24 hour treatment with 10-4M TAM (Lane 2), 10-4M E2 (Lane 3), 10-6M TAM (Lane 4), 10-6M E2 (Lane 5), and in 0.1% DMSO control (Lane 6).

DISCUSSION

Tamoxifen, a nonsteriodal triphenyl ethylene has structural conformation such that it resembles steroidal molecules located within the nucleus of cells. This enables it to attach to the ER receptors, and thus antagonize against molecules as estrogens (Skidmore et al., 1972; Duan et al., 1998 and 2001). Tamoxifen has also served as a useful anti-estrogen in the treatment of human breast cancer for more that twenty years (Heel et al., 1978; Osborne et al., 1984; Powel, 1997). Therefore it follows logically that tamoxifen is able to inhibit cell growth and induce apoptosis. Various extra cellular signaling molecules can upregulate or downregulate mammalian cell proliferation via G_1-targeting pathways (Jordan and Prestwich, 1977; Draetta, 1994; Sherr, 1996). It has long been recognized that tamoxifen possesses growth inhibitory properties in human breast cancer cell growth

and that this can occurs as a result of disrupting the cell cycle as a result of provoking estrogen receptor stimulation (Prall et al., 1997).

We have demonstrated that the acute administration of estradiol and tamoxifen for even a shorter period of time does induce regression/alteration of various phases of the cell cycle. More specifically, there was a decline in the S phase and G_2/M phases with relatively higher concentrations of 17β-estradiol (10^{-4}M), and tamoxifen (10^{-4}M) resulting in reductions in G_0/G_1, S and G_2/M phases. MCF-7 cells are known to be estrogen responsive (DePasquale et al., 1994). Tamoxifen has been known to be an inducer of apoptosis in a dose-dependent manner (Ercoli et al., 1998), and also known is the ability of estrogens to appear anti-proliferative in its induction of MCF-7 growth at higher concentrations of administration (Safe, 1998). The data gathered in this experiment are supported by the previously published reports that estrogens have a direct effect on the growth of human mammary tumor cells (Horwitz and McGuire, 1978; Lippman, 1981; Coezy et al., 1982; Darbe etal., 1983). The possibility of estradiol dependent growth resulting from the residual estrogens present in serum may be excluded because indigenous estrogens were supposedly removed by using charcoal-dextran treated serum in the preparation of MCF-7 cells.

When the two concentrations were combined, i.e.; 17β-estradiol and tamoxifen (both 10^{-4}M), the G_0/G_1 phase DNA was decreased in MCF-7 cells. However, that did not occur with 17β-estradiol (10^{-4}M) only treatment; but tamoxifen (10^{-4}M) alone was able to reduce G_0/G_1 phase DNA. This is a feasible indicator that at this concentrations, the tamoxifen molecule is the stronger and more successful competitor for the ERα receptor site.

Additionally, we have indicated that 10^{-4}M tamoxifen altered MCF-7 cell morphology, as well as downregulated F-actin very severely. Then, 10^{-4}M 17β-estradiol also changed cell shape and decreased actin filament protein moderately (not as severely as 10^{-4}M tamoxifen). Here, we see a direct relationship between tamoxifen (Porter and Janicke, 1999) and estrogens (Horwitz and McGuire, 1978; Lippman, 1981; Coezy et al., 1982; Darbe etal., 1983) as it relates to inhibitory effects of cell proliferation of MCF-7 growth. It is therefore possible that the downregulation and possible cleavage or depolymerization of F-actin is instrumental in cell growth inhibition and even apoptotic cell death (Bursch et al., 2000). Tamoxifen, at relatively higher concentrations induced apoptosis in MCF-7 cells (Ogba et al., 2005). The elevated G_2/M phase as observed with 10^{-4}M 17β-estradiol treatment could be indicative of the G_2/M checkpoint attempting to accommodate the reduction of genetic material within the cell due to reduced DNA synthesis resulting from a depressed S phase.

In another study in which MCF-7 cells were treated with indol-3-carbinol (I3C) acid-catalyzed derivatives, growth suppression was observed (Cover et al., 1999). It was noted that suppression was preceded by an arrested G1 and that this reversible disruption was accompanied by a downregulation of CDK6 (Cover et al., 1998). This I3C study although independent of estrogen receptor signaling indicated the intrinsic role of CDKs in cell cycle alteration. However in an estrogen receptor signaling related study, it was noted that estrogen-induced activation of CDK4 as well as CDK2 occurring while human mammary cancer cells are progressing from the G1 to the S phase was accompanied by an up regulated cyclin D1 expression and lower associations between CDK inhibitors and the cyclin E-CDK2 complex (Prall et al., 1997). It is therefore not unreasonable to

propose that CDK expression may be directly or indirectly affected by the action of higher concentrations of tamoxifen and 17β-estradiol on estrogen receptors. In a recent study done *in vitro* on mice, it was discovered that there exists a positive correlation between CDK and CKI [p27 (kip1)] in cells in G0-arrest that contributes to regulating cytoskeletal reorganization in neuronal processes. The study utilized RNA interference (RNAi) to demonstrate that decreased quantities of p27 reduced the quantity of F-actin found in migrating neural processes (Kawauchi et al., 2006). It is therefore feasible to postulate that CDK disruptions (and possible cell cycle alterations) can have effects on F-actin expression. Hence, this may be one possible pathway through which the interaction of tamoxifen and estrogens with ERα receptors can trigger the down regulation of F-actin as seen in the present study.

In conclusion, the current study indicates a possible relationship between the down regulation of F-actin expression and the ability of tamoxifen and 17β-estradiol to induce differentiations and alterations in MCF-7 cell cycle progression. This provides a useful model to study post-translational modification of F-actin regulation of cytoskeleton in ER responsive breast cancer tumor cells.

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