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13. ABSTRACT ***(Maximum 200 Words)***

The overall goal of this research is to determine whether the selective estrogen receptor modulator (SERM), tamoxifen can induce a metastatic phenotype in non-invasive estrogen receptor positive (ER+) MCF-7^{RFP} cells grown as orthotopic xenografts in nude mice. The experiments in this proposal were designed to compare the spread of MCF-7^{RFP} with that of a well characterized estrogen receptor negative metastatic cell line MDA-MB0-345 tagged with green fluorescent protein. Pilot studies have shown that tamoxifen can induce the metastatic spread of MCF-7^{RFP} at least as far as the lymph nodes. Due to morbidity problems unrelated to tumor progression, the major experiment was stopped before it was possible to establish whether MCF-7^{RFP} can also metastasize to other more distant organs such as the ling, and liver as is seen with the MDA-MD-435^{GFP} cells. These data suggest that tamoxifen can induce lymphatic spread of non-invasive cell line and promotes the survival of the tumor cells in the lymphatic system. We have not yet established whether hematogenous spread of the ER+ cells can also occur.

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INTRODUCTION: Selective Estrogen Receptor Modulators (SERMs), such as tamoxifen, have been used for many years as hormonal therapy for advanced breast cancer (Furr and Jordan 1984; Love, 1989). Tamoxifen is currently being evaluated both for the treatment of early, organ confined breast cancer and for chemoprevention for women at high risk for the disease. In the course of *in vitro* studies, we and others have shown that tamoxifen exerts its effects on estrogen receptor positive MCF-7 cells by inducing apoptosis in the majority of cells (Perry et al, 1995; Bursch et al., 1996; Simboli-Campbell et al., 1996). However, a small proportion of the cells fail to die and acquire an invasive phenotype (Gilmore and Tenniswood, unpublished data). This phenomenon is not seen when MCF-7 cells are treated with other agents (such as TNFα, or the pure anti-estrogen ICI 182,780) that also induce apoptosis in these cells, suggesting that tamoxifen induces a unique pattern of gene expression in these cells. The experiments supported by this Concept award are designed to test the ability of tamoxifen, and ICI 182,780 to induce metastatic progression in a preclinical model of breast cancer. Breast cancer cells stably transfected with Red or Green Fluorescent Protein (GFP) have been grown as orthotopic implants in nude mice and then treated with tamoxifen periods so far ranging up to 8 weeks. At defined time points throughout the treatment we will continue to examine the effects of the tamoxifen and other SERMs on the growth or regression of the primary tumor and on the metastatic progression to other tissues, using fluorescent microscopy to determine the number and sites of metastases originating from treatment with each drug, and monitor the time at which the metastases arise. These data will directly answer the question as to whether the SERMs induce a metastatic phenotype in this pre-clinical model of localized breast cancer.

BODY: (*The information presented on pages 4 through 9 represent unpublished data that should be protected*)

Timeline of the experiments:

As outlined below in more detail the completion of the current study has been significantly handicapped due to a long back order of female nude mice from Taconic Farms, and a subsequent Staphylococcal infection in the nude mice mice utilized for this study. The first phase of the animal study was planned to start in August 2001, but the animals were back ordered for 2 months at that time. These animals were used in a pilot study to compare the growth and metastatic spread of MCF-7 cell transfected with red fluorescent protein (MCF-7^{RFP}) and MDA-MB-435 cells transfected with Green Fluorescent protein (MDA-MB-435^{GFP}), in ovariectomized nude mice in the absence or presence of 1.7 mg sustained release estradiol pellets. These studies were designed to demonstrate that the fluorescently tagged cells would retain their appropriate responsiveness to estrogen when grown in nude mice, and that the fluorescence could be used to track the tumor cells in the primary tumors and to metastatic sites. The pilot study was successfully completed in January 2002.

The second major experiment was designed to compare the growth and metastatic potential of MCF-7^{RFP} cells treated with anti-estrogens, to the well characterized metastatic breast cancer cell line MDA-MB-431^{GFP}. This study was initiated in April 2002. The animals in this study (120 total) were ovariectomized by the suppliers implanted with placebo pellets, 0.72 mg or 1.7 mg sustained release estradiol pellets (Innovative Research), shipped to the Freimann Animal Care facility at the University of Notre Dame, and inoculated with either MCF-7^{RFP} or MDA-MB-435^{GFP} cells and subsequently implanted with tamoxifen sustained release pellets (Innovative Research), using exactly the same protocol as outlined in the pilot experiment. Primary tumor growth was essentially the same as seen in each group the Pilot experiment. However the mice in the group receiving high levels of estradiol (via 1.7 mg sustained release pellets) developed a substantial Staphylococcal infection (see Figure 3), and all but 5 of the 40 had to be sacrificed due to advanced morbidity and weight loss. The decision was made to terminate the study in August, 2002, and to set up a repeat study immediately. While this rendered the experiment statistically invalid, and necessitates repetition to test the hypothesis, some interesting data has emerged from the study based on the surviving animals which were sacrificed after 5 months (see Figure 2).

As a result of these delays, we have asked for, and received, a one year no cost extension in May 2002, and the repetition of the major experiments was initiated at the beginning of September, 2002. Based on our preliminary findings, this experiment should be completed This experiment is expected to produce enough data for analysis in February 2003 and to be completed by September 2003.

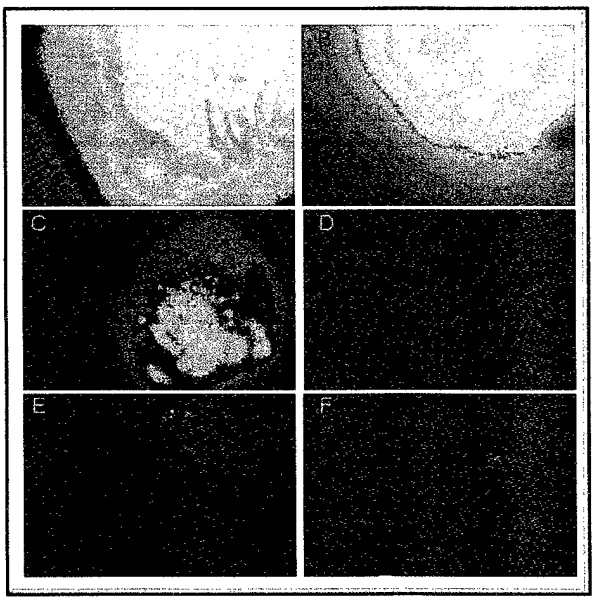

Figure 1: Green Fluorescent Protein effectively marks the metastatic MDA-MB-435 cells in the primary tumor (Panel A); the lymph nodes (Panel B); the wall of the peritoneal cavity (Panel C); rib cage (panel D); Lung (Panel E) and Liver (Panel F).

Results to date:

Pilot Experiment

As shown in Figure 1, we have shown that using green fluorescence, it is possible to trace the metastatic spread of MDA-MB-435^{RFP}, estrogen receptor negative (ER-) tumor to the lung, lymph nodes, liver, rib cage and peritoneal cavity.

MCF-7^{RFP} tumors on the other hand form large primary tumors with clearly defined margins which do not appear to metastasize at all. The morphology of the primary tumors was assessed was assessed and we have shown that the primary tumors formed by the RFP over-expressing

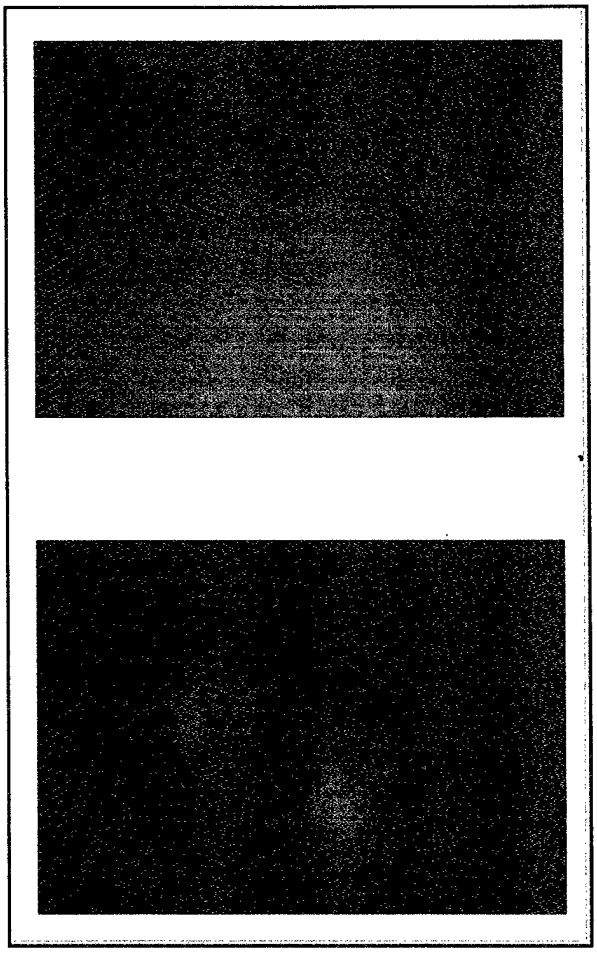

Figure 2: RFP fluorescence of MCF-7 cells in the primary tumor (top panel) and in the thoracic lymph nodes of a mouse treated with tamoxifen in the presence of estradiol.

MCF-7 cells are virtually indistinguishable from the tumors formed by the untransfected parental cells.

Major study Comparison between the metastatic potential of untreated MDA-MB-435 cell with MCF-7^{RFP} tumors treated with Tamoxifen. While animal losses from this experiment preclude any meaningful data analysis, one of the five animals that were implanted with 1.7 mg estradiol and treated with tamoxifen showed clear evidence of metastatic spread to the lymph nodes (see Figure 2). These data provide very tantalizing suggestion that the non-invasive, ER+ cell line can become metastatic when treated with tamoxifen, particularly since the orthotopic tumors were established in the inguinal fat pads and these red fluorescing metastases were found in the ipsilateral thoracic lymph nodes. These data strongly suggest that the MCF-7 cells are capable of lymphatic spread after tamoxifen treatment, but since these animals were sacrificed after 5 months due to morbidity related to the Staphloccocal infection, it is not possible to determine whether the cells are capable of spreading to other organ sites in a manner reminiscent of the MDA-MB-435 cells. It should be noted here that this pattern was seen in 1/5 animals in the group implanted with 1.7 mg pellets releasing a high level of estradiol, but has not been seen in the animals implanted with 0.72 mg sustained release pellets that produce a significantly lower serum estradiol level and induce slower tumor growth.

Additional Data Related to the in vivo Study

In the course of these studies it became apparent that MDA-MB-321 cells respond to tamoxifen *in vivo* , and some tumor regression occurs, even though the tumors are classified as estrogen receptor negative. In a related set of *in vitro* experiments, performed concurrently by Dr. Louise Flanagan who is responsible for the *in vivo* studies and Dr. Kerry Gilmore, another post-doctoral fellow in the laboratory, we have established that these cells express ERβ, and that tamoxifen

induces apoptosis in these cells through this receptor via a caspase independent independent pathway. As listed below in the Reportable Outcomes, this work was presented at two Symposia, including the Era of Hope Meeting in Orlando and has been accepted for publication by the Journal of Steroid Biochemistry and Molecular Biology.

Nature of the Staphylococcal Infection

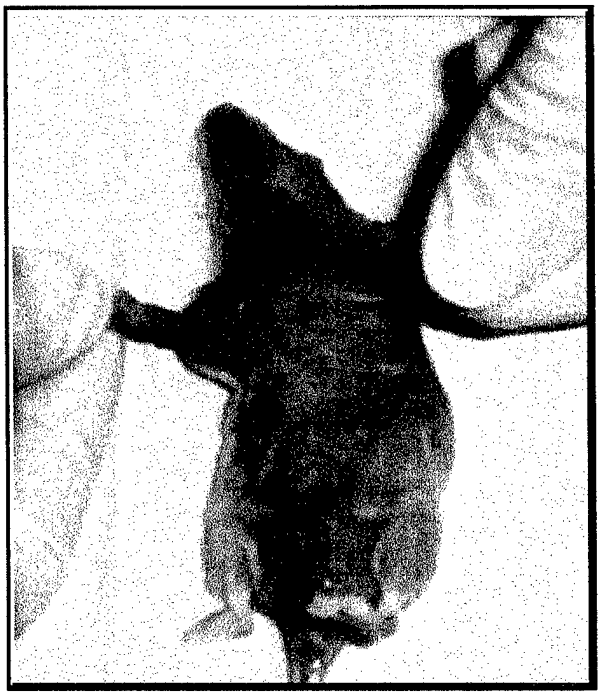

Figure 3: Female mouse, supplemented with 1.7 mg sustained release estradiol pellets carrying an orthotopic implant of MCF-7^{RFP} cells, showing advanced signs of Staphylococcus infection on forlegs.

The nude mice implanted with 1.7 mg sustained release eatsradiol pellets slowly developed a crusty, weeping infection under the forelegs and on the chin (Figure 3). The animals lost weight (up to 5 grams), triggering the criteria that necessitates ending the study. The pathology of these mice, and the nature of the infection was determined in consultation with the School of Veterinary Medicine at Purdue University, West Lafayette IN. It is not yet clear whether the source of the bacteria infection is the supplier of the mice (Taconic), the supplier of the pellets (Innovative Research) or our handling of the animals. The fact that similar infections have been noted in other nude mice from the same supplier purchased on another day , suggests that problems probably originate with the supplier, who has agreed to supply the animals for the repeat experiment without cost.

KEY RESEARCH ACCOMPLISHMENTS

The final outcome of the repeat studies has not been determined as yet, although there is a clear preliminary indication that tamoxifen induces metastatic spread of MCF-7 cells at least to the lymph nodes. These data suggest that tamoxifen can induce lymphatic spread of ER+ non-metastatic cells. These preliminary observations will be confirmed in the repeat of the study that is now underway, and with a complete study may demonstrate the hematogenous spread of these cells to other metastatic sites such as the lung and liver. If these findings demonstrate that the tamoxifen induces metastatic spread only to the lymph nodes, it will raise an important distinction between lymphatic spread of the disease and

hematogenous spread from the primary tumors. Whether these differences arise because of the differences in lymphangiogenesis and angiogenesis, or due to differences in the ability of the MCF-7 cells to survive in the lymphatics versus the circulation remains to be determined.

REPORTABLE OUTCOMES

1. Presentations

Work emanating from the experiments related to the in vitro characterization of the ability of tamoxifen to induce apoptosis in ER- MDA-MB-435 and SUM 159PT has been presented at two International Meetings:

Gilmore K, Flanagan L, Wagner C, Hurley J, and Tenniswood M Induction of Apoptosis in Estrogen Receptor Negative Human Breast Cancer Cells by the Anti-estrogen Tamoxifen. (Abstract of Poster presentation at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, May 2002 Munich, Germany)

Tenniswood M, Gilmore K, Flanagan L, Wagner C, and Hurley J. Mechanistic Comparison of the Induction of Cell Death in Estrogen Receptor Negative Breast Cancer Cells. (Abstract 10-27, ERA of HOPE, Department of Defense Breast Cancer Research Program Meeting September, 2002 Orlando FL).

2. Manuscripts

One manuscript describing the induction of apoptosis in ER- breast cancer cells by tamoxifen been accepted for publication:

Flanagan L, Gilmore K, Wagner C, Hurley J and Tenniswood M. (2003) Induction of Apoptosis in ER Negative Breast Cancer Cells by the Selective Estrogen Receptor Modulator Tamoxifen (accepted, Journal of Steroid Biochemistry and Molecular Biology)

3. Experimental Materials Generated

In the course of these studies we have engineered ER+ MCF-7 cells that express green fluorescent protein and red fluorescent protein (MCF-7^{GFP} and MCF-7^{RFP} cells respectively) well as ER- SUM-159PT and MDA-MB-435 cells that express these fdluorescent tags. These cell lines have been distributed to a number of researchers under a non-restrictive Material Transfer Agreement. To our knowledge at least one manuscript has been submitted utilizing one or more of these cell lines (Iyengar, P et al., submitted).

CONCLUSIONS

Due to back-ordering of animals and a chronic Staphyococcal infection these major study has yet to be completed. The preliminary *in vivo* data and the accompanying *in vitro* experiments however suggest that the hypothesis being tested, that tamoxifen treatment can induce an invasive/metastatic *in vivo,* is valid. We expect the repeat experiment to provide definitive data that can test the hypothesis in a statistically valid way.

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APPENDIX

Induction of Apoptosis in ER Negative Breast Cancer Cells by the Selective Estrogen Receptor Modulator Tamoxifen.

Louise Flanagan*, Kerry Gilmore*, Caroline Wagner, Jennifer Hurley and Martin Tenniswood

* Both Authors contributed equally to this work

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Portions of this work were presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Munich, Germany, May 2002. Supported by Post-doctoral fellowships from the Susan G. Komen Foundation (LF), the Department of Defense Breast Cancer Research Program (LF) and the Walther Cancer Institute (KG). The research was funded by operating grants from the Department of Defense Breast Cancer Research Program in the form of a Concept award and by an unrestricted operating grant from the Coleman Foundation.

ABSTRACT

Tamoxifen, a selective estrogen-receptor modulator (SERM) has been used for the treatment of all stages of hormone-responsive breast cancer for more than 25 years. The primary mechanism of action of tamoxifen is believed to be through the inhibition of estrogen receptor alpha (ERα), however the clinical observation that up to 30% of ER-negative breast cancers respond to tamoxifen treatment suggests that additional non-ER-α mediated mechanisms exist. To elucidate the intracellular mechanism of tamoxifen action in ER-negative tumors we have utilized two ER-α negative human breast cancer cell lines: SUM-159PT and MDA-MB-435 cells. Both the SUM-159PT and MDA-MB-435 cells are growth inhibited by tamoxifen in a time dependent manner, through the induction of G_0/G_1 and G_2M growth arrest and apoptosis at levels similar to that observed in the MCF-7 cells. ICI 182,780, a pure anti-estrogen, which induces cell cycle arrest and apoptosis in ER-α positive MCF-7 cells, does not induce these effects in the ER-α negative breast cancer cell lines. Tamoxifen mediated apoptosis is associated with translocation of the pro-apoptotic protein Bax to mitochondria, release of cytochrome c and high molecular weight DNA fragmentation in both the ER-α-negative and positive breast cancers cells suggesting that tamoxifen induces a common apoptotic pathway. Furthermore caspase-3 is activated in MDA-MB-435 cells after treatment with tamoxifen. Both SUM-159PT and MDA-MB-435 express the newly identified estrogen receptor, estrogen receptor-beta (ER-β) at levels similar to that observed in MCF-7 cells suggesting that tamoxifen may exert its effects through this receptor.

KEYWORDS

Estrogen receptor-alpha, Estrogen receptor-beta, tamoxifen, ICI 182,780, apoptosis, breast cancer

INTRODUCTION

Tamoxifen, a selective estrogen modulator (SERM), and ICI 182,780, a steroidal pure anti-estrogen, have been shown to induce cell cycle arrest and apoptosis in estrogen dependent breast cancer [1-9]. These compounds interact with the estrogen receptor (ER), altering its conformation and consequently changing either its DNA binding ability (in the case of ICI 182,780) or by altering the interactions of the ER with accessory proteins, (in the case of tamoxifen) [10].

In patients diagnosed with breast cancer, the presence of the ER is a good prognostic indicator, identifying patients with a lower risk of relapse and better overall survival [11-15]. 70% of ER-positive patients respond to anti-estrogen therapy [16, 17]. However somewhat paradoxically tamoxifen also induces an objective response in a significant proportion of ER-negative breast cancers and several other malignancies including hepatocellular, ovarian, pancreatic and renal cell cancer [7, 18-22]. Recent *in vitro* studies have demonstrated that tamoxifen induces the morphological and biochemical changes that are characteristic of apoptosis in ER-negative MDA-MB-231 and BT-20 human breast cancer cells at μM concentrations [23]. Animal model systems have shown significant inhibition of angiogenesis by tamoxifen in the treatment of ER-negative fibrosarcomas and suggest possible contributory mechanism of ER-independent manipulation by tamoxifen in the treatment and prevention of breast cancer. [24] Although several signaling intermediates such as protein kinase C (PKC), transforming growth factor -β (TGF-β), JNK1 and c-myc have been implicated the precise molecular mechanism of tamoxifen induced apoptosis remains unclear [5, 18, 23, 25-27].

The recent identification of a second ER gene, designated ER-β to distinguish from the classical ER which has been renamed ER-α, offers a possible mechanism of action for tamoxifen and other SERMS. The two isoforms of the receptor share 95% homology in their DNA binding region and 55% homology in the ligand binding domain but have little homology in other regions. [28, 29]. ER-β has been identified in both normal and malignant mammary glands and ER-β positive tumors are associated with low histological grade, negative axillary nodes and low S-phase fraction, indicating that they may be less aggressive and more responsive to hormonal

intervention [30-33]. Furthermore in women treated with adjuvant tamoxifen therapy, expression of ER-β in more than 10% of cancer cells was associated with better survival suggesting that ER-β status is a significant predictor of reponse to adjuvant tamoxifen therapy in women with breast cancer [34].

Antagonistic effects of tamoxifen were previously thought to be mediated through ER-α, however it has recently been shown that while tamoxifen is a partial agonist of ER-α it is a pure antagonist of ER-β [35, 36]. Estradiol upregulation of the ER-β mRNA in T47D cells can be abolished by anti-estrogens such as tamoxifen [30]. Thus ER-β expression may serve as a prognostic, diagnostic and or therapeutic marker for breast cancer.

These findings, and the fact that a subset of ER-negative breast cancers and other ER-negative cancers respond to tamoxifen prompted us to compare the effects of tamoxifen and the pure anti-estrogen ICI 182,780 on an ER-α-positive MCF-7 breast cancer cell line and two ER-α-negative breast cancer cell lines; SUM-159PT – a newly characterized ER-negative cell line and MDA-MB-435 [37]. The data presented suggest that tamoxifen induces a time dependent decrease in cell growth in both ER-α-positive MCF-7 and ER-α-negative SUM-159PT and MDA-MB-435 cells, but the growth inhibitory and apoptotic effects of ICI 182,780 is limited to the ER-α-positive MCF-7 cells. Tamoxifen induced decrease in cell growth is associated with morphogical and biochemical markers of apoptosis including bax translocation to the mitochondria, cytochrome c release, high molecular weight DNA fragmentation and selective activation of caspases. Our results also show the expression of ER-β mRNA in both ER-α negative and positive cells, indicating a possible mechanism for the effects mediated by tamoxifen in ER-α-negative breast cancer cells. Further elucidation of the role of ERβ in breast cancer progression and sensitivity to tamoxifen is warranted.

MATERIALS AND METHODS

Cell culture and growth assays

MCF-7 cells (ER-positive, non-invasive) were obtained from ATCC, SUM-159PT cells (ER-negative, invasive) were obtained from the University of Michigan Breast Cancer Cell/Tissue Bank and the MDA-MB-435 cells (ER-negative, invasive) were obtained from the Lombardi Cancer Center. The cell lines were cultured in α-MEM medium (Life Technologies Inc, Gaithersburg, MD) containing 25mM HEPES and 5% fetal bovine serum (ATLAS Biologicals, Fort Collins, CO). Cells were routinely passaged every 4-5 days. For cell growth and morphological assays, MCF-7 cells were plated at 10,000 cells/well in 24-well plates; SUM-159PT and MDA-MB-435 cells were plated at 8,000 cells/ well in 24-well plates, and treated with 10 μM tamoxifen (Sigma, St. Louis, MO), 10 μM ICI 182,780 (Astra-Zeneca, Macclesfield, UK) or ethanol vehicle two days after plating for the indicated times. Cell growth was determined by crystal violet assays. For flow cytometry, subcellular fractionation and caspase assays MCF-7 cells were plated at a density of 8×10^5 cells/150-mm flask. SUM-159PT and MDA-MB-435 cells were plated at a density of 6×10^5 cells/150-mm flask. 48 h following plating, cells were treated with ethanol vehicle, 10 μM tamoxifen,10 μM ICI 182,780 or 1 μM or 5μM etoposide for the indicated times.

Immunohistochemistry

Cells grown on Lab-Tek II chamber slides (Fisher Scientific) were treated with 10 μM tamoxifen, 10 μM ICI 182,780 or ethanol vehicle two days after plating. The cells were fixed in 4% formaldehyde (Fisher Scientific) in PBS for 5 min at room temperature and permeabilized in methanol for 5 min at -20°C. DNA fragmentation was assessed by TdT-mediated dUTP nick end labeling (TUNEL) using a commercially available assay according to manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, IN) and detected using an anti-fluorescein antibody conjugated with horse-radish peroxidase (POD) followed with diaminobenzidine (DAB; Sigma) to

localize peroxidase. Cells were counterstained with hematoxylin and photographed using an Olympus AX70 microscope equipped with a Spot RT digital camera.

Flow Cytometry

For analysis of DNA fragmentation and cell cycle kinetics, cells were harvested by trypsinization, pooled with floating cells, pelleted by centrifugation (500 g, 5 min, 4°C), fixed and permeabilized with 90% EtOH at -20°C. DNA strand breaks were labeled with bromodeoxyuridine (BrdU) by terminal transferase (Roche Molecular Biochemicals) and detected using a FITC-conjugated anti-BrdU monoclonal antibody (Phoenix Flow Systems, San Diego, CA). Cells were counterstained with 5 μg/ml propidium iodide (PI; Sigma) containing RNase A (Roche Molecular Biochemicals). DNA strand breaks and cell cycle were analyzed by flow cytometry using an Epic XL Flow Cytometer (Coulter Corp., Miama, FL). Data was analyzed using Multiplus software (Phoenix Flow Systems).

Subcellular fractions

Adherent cells were trypsinized, pooled with floating cells and pelleted by centrifugation (500 g, 5 min, 4°C), resuspended in wash buffer (25 mM Tris pH 7.5, 250 mM sucrose, 2.5 mM $MgCl_2$, protease and phosphatase inhibitors), pelleted (500 g, 5 min, 4°C), resuspended in 3 volumes of buffer A (20 mM HEPES-KOH 0.5 M, pH 7.5, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose protease and phosphatase inhibitors), dounce homogenized, centrifuged (500 g, 5 min, 4°C) and the supernatants transferred to an ultracentrifuge tube. The pellets (containing nuclei) were resuspended in 0.5 vol Buffer A and re-centrifuged (500 g, 5 min, 4°C). The combined supernatants were centrifuged (100,000 g, 1 h, 4°C) in a Beckman ultracentrifuge to generate the cytosolic fraction (supernatant) and the non-nuclear membrane fraction (NNMF) (pellet). The pellets were resuspended in 100 μl Buffer A, sonicated and analyzed for total protein using the Micro BCA protein assay (Pierce, Rockford, IL).

Western blot analysis

Subcellular fractions were solubilized in Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. Proteins derived from the NNMF and/or cytosolic extracts were immunoblotted with a rabbit polyclonal antibody directed against Bax (Pharmingen, San Diego, CA) or a mouse monoclonal antibody directed against cytochrome c (Pharmingen) diluted 1:250 and 1:100 respectively in PBS plus 5% skimmed milk. Specific antibody binding was detected by appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Pitcataway, NJ) diluted 1:5000 in PBS plus 5% skimmed milk and detected using enhanced chemiluminescence. (Pierce).

Field Inversion Gel Electrophoresis

Field inversion gel electrophoresis (FIGE) was carried out on a Chef Mapper XA Pulsed Field Electrophoresis System (Bio-Rad Labs, Hercules, CA). 2×10^6 cells were embedded in 1% low melt agarose (Promega, Madison, WI) plugs and proteinase K digestion was carried out as described [38]. Electrophoresis was conducted at 14°C in 0.5 x TBE at 150 V (forward and reverse). The forward switch time increased linearly from 5.4 s to 54 s and the reverse switch time increased linearly from 1.8 s to 18 s over the 19 h run time. The gel was stained for 1 h in 1 μg/ml ethidium bromide, destained in water and photographed using a Kodak Electrophoresis Documentation and Analysis System 120. λ-DNA ladder was obtained from BioRad Labs.

Caspase Activation Assays

The relative levels of caspase activity in cell lysates were determined using a fluorescence microplate assay. Cell extracts were produced by repeated freeze-thawing under reducing conditions, followed by centrifugation (8,000 x g, 15 min, 4°C). The fluorogenic substrates DEVD.AMC (10 μM), IETD.AFC (50 μM) and LEHD.AFC (250 μM) were used to measure the activation of caspases -3, -8, and -9 respectively. The specificity of activation was determined by comparison with fluorescence levels generated in the presence of caspase

inhibitors DEVD.CHO (1 μM), IETD.CHO (10 μM) or LEHD.CHO (100 μM) for caspases -3, -8 and -9 respectively. Fluorescence emission was measured at 460 nm for caspase-3 or 510 nm for caspases -8 and -9 after excitation at 390 nm.

Reverse-transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Life Technologies) according to manufacturers protocol. First strand cDNA was synthesized utilizing oligo(dT) primers and Superscript II RNase H⁻ Reverse Transcriptase (RT) (Life Technologies) as previously described [39]. Amplification of ER-α and ER-β cDNA was carried out using primers previously described [39]. The cDNA was amplified directly using primers specific for ER-α, ER-β and GAPDH to generate PCR products of 483bp, 242bp and 680bp respectively.

For ER-α the primers were as follows:

5' primer: 5' - CAG GGG TGA AGT GGG GTC TGC TG -3'

3' primer: 5' - ATG CGG AAC CGA GAT GAT GTA GC - 3'

The amplification conditions for the ER-α PCR were as follows: A denaturation step for 2 min at 94°C, followed by 1 min at 94°C, 45 sec at 68°C and 1 min at 72°C for 35 cycles, followed by 10 min at 72°C. 20 μl of the PCR reaction product was analyzed on a 1.5% agarose gel.

For ER-β the primers were as follows:

5' primer: 5' - GTC CAT CGC CAG TTA TCA CAT C - 3'

3' primer: 5' - GCC TTA CAT CCT TCA CAC GA - 3'

For GAPDH the primers were as follows:

5' primer: 5' - CAT CTC TGC CCC CTC TGC - 3'

3' primer: 5' - CTC TTC CTC TTG TGC TCT TGC - 3'

The amplification conditions for the ER-β and GAPDH PCR were as follows: A denaturation step for 2 min at 94°C, followed by 1 min at 94°C, 0.5 min at 60°C and 1 min at 72°C for 35 cycles, followed by 10 min at 72°C. 20 μl of the PCR reaction product was analyzed on a 1.5% agarose gel.

Statistical Evaluation

Statistical analyses were performed using GraphPad Instat (GraphPad Software, San Diego, CA). Data are expressed as Mean ± SEM. One way analysis of variance was used to assess statistical significance between means. Differences between means were considered significant if p values less than 0.05 were obtained using the Bonferroni method.

RESULTS

Effects of tamoxifen and ICI 182,780 on cell growth and cell cycle progression in ER-positive and ER-negative cell lines

To determine whether anti-estrogens affect ER-negative breast cancer cells, the effects of tamoxifen and ICI 182,780 on cell growth of ER-positive MCF-7 and ER-negative SUM-159PT and MDA-MB-435 cells were compared over a 72 h time course (Figure 1). In MCF-7 cells, tamoxifen clearly induces cell death, initiating a significant decrease in cell number compared to vehicle controls at 24 h. ICI 182,780 on the other hand appears to induce cell cycle arrest (Table 1) without inducing significant cell loss at 24 h, suggesting that the drug is cytostatic rather than cytotoxic. Tamoxifen also induces apoptosis in SUM-159PT and MDA-MB-435 cells even though these cells are classified as ER-negative (Figure 1). ICI 182,780 does not induce either apoptosis or cell cycle arrest in SUM-159PT or MDA-MB-435 cells, since cells treated with this compound grow with essentially the same kinetics as the control cells (Table 1).

As summarized in table 1, tamoxifen induces G_0/G_1 arrest in MCF-7, SUM-159PT and MDA-MB-435 cells and appears to increase the G_2M fraction in MDA-MB-435 cells, suggesting that the drug facilitates entry into S phase but induces a subsequent block in replication. While ICI 182,780 induces G_0/G_1 arrest in MCF-7 cells, it has no effect on cell cycle progression in ER-negative breast cancer cell lines.

Induction of cell death by tamoxifen but not ICI 182,780 in ER-negative cell lines

The ability of tamoxifen and ICI 182,780 to induce cell death in MCF-7, SUM-159PT and MDA-MB-435 cells was assessed by TUNEL labeling (Figure 2A) and flow cytometry (Figure 2B). Both tamoxifen and ICI 182,780 induce apoptosis in ER-positive MCF-7 cells (Figure 2A). However, whereas tamoxifen induces apoptosis in SUM-159PT and MDA-MB-435 cells (as evidenced by decreased cell number and positive TUNEL staining of the remaining cells), cells treated with ICI 182,780 show little or no evidence of DNA fragmentation after 48 h of treatment. Quantitation of the degree of fragmentation in the cell lines after treatment with tamoxifen or ICI

182,780 confirms that both SUM-159PT and MDA-MB-435 cells show evidence of significant DNA fragmentation (27% and 26% BrdU positive cells respectively, compared to 43% for MCF-7 cells) after treatment with tamoxifen. Neither ER-negative cell line shows evidence of DNA fragmentation after treatment with ICI 182,780. Tamoxifen and ICI 182,780 treatment of MCF-7 cells induced DNA fragmentation primarily in the G_0/G_1 phase of the cell cycle; SUM-159PT cells induced DNA fragmentation in the G_0/G_1 phase of the cell cycle after treatment with tamoxifen while MDA-MB-435 cells induced DNA fragmentation in the G_0/G_1 and G_2M phases of the cell cycle (data not shown).

Disruption of mitochondrial function by tamoxifen in MCF-7, SUM-159PT and MDA-MB-435 cells

To elucidate the intracellular events involved in tamoxifen mediated apoptosis, the apoptotic signaling pathways induced by tamoxifen in MCF-7, SUM-159PT and MDA-MB-435 cells were determined. Etoposide, an established chemotherapeutic drug that induces apoptosis via inhibition of topisomerase II was used as a positive control. Since disruption of mitochondrial function is a primary event in apoptosis that is triggered by Bax translocation to mitochondrial outer membranes, subcellular distribution of Bax after tamoxifen treatment was examined in these cell lines. Bax redistribution from the cytosol to the mitochondria and cleavage to form the 18 kDa active t-Bax fragment occurs after treatment with 10 μM tamoxifen (Figure 3A). Translocation of Bax to the mitochondria has been associated with release of cytochrome c, an event considered a commitment point for the activation of apoptosis. As expected in control cultures, cytochrome c is only detected in the mitochondrial fraction (data not shown). Redistribution of cytochrome c from mitochondria to cytosol is detected after 48 h in tamoxifen and etoposide treated MCF-7, SUM-159PT and MDA-MB-435 cells (Figure 3B), although the amount of cytochome c recovered from the cytosol of SUM-159PT cells is significantly less than MCF-7 or MDA-MB-435 cells.

Caspase activation in SUM-159PT and MDA-MB-435 cells following treatment with tamoxifen, ICI 182,780 or etoposide

Neither tamoxifen or ICI 182,780 induce the activation of caspases-3, -8 or -9 in SUM-159PT cells, even though it is clear that the enzymes are present in these cells and are activated by treatment with etoposide (Figure 4A). In contrast, tamoxifen induces significant levels of capase-3 activity (which peaks 24 h after treatment) as well as transient induction of caspase-8 and -9 at 12 h. ICI 182,780 also appears to transiently induce caspases-3 and -8 at 12 h in these cells although the degree of induction is minimal (Figure 4B).

Induction of DNA fragmentation by tamoxifen and ICI 182,780 in ER-negative breast cancer cell lines.

As shown by field inversion gel electrophoresis (FIGE), tamoxifen induces significant DNA fragmentation to 450-600 Kbp and 50-100 Kbp in MDA-MB-435 cells (Figure 5). ICI 182,780 and etoposide also induce DNA cleavage to form high molecular weight fragments but at levels that are significantly lower than that of tamoxifen treated cells. SUM-159PT cells undergo similar patterns of DNA fragmentation after treatment with tamoxifen, ICI 182,780 and etoposide (data not shown). Standard agarose gel electrophoresis has demonstrated that there is no further fragmentation of the DNA to oligonucleosomes in the ER-negative cell lines (data not shown).

Expression of ER-α and ER-β expression in MCF-7, SUM-159PT and MDA-MB-435 breast cancer cells

ER-α and ER-β expression in MCF-7, SUM-159PT and MDA-MB-435 cells was assessed by RT-PCR. The expected ER-α PCR product of 242 bp is amplified strongly in MCF-7 cells but not observed in ER-negative MDA-MB-435 and SUM-159PT breast cancer cells. However, the expected ER-β PCR product of 483 bp is amplified in all three breast cancer cell lines. Standardization of the signal using the 680 bp GAPDH PCR product suggests that ER-β mRNA expression is highest in MCF-7 cells and lowest in MDA-MB-435 cells.

DISCUSSION

Both tamoxifen and ICI 182,780 have been shown to induce cell cycle arrest, high molecular weight DNA fragmentation, loss of mitochondrial membrane potential and apoptosis in estrogen-dependent breast cancer cells including MCF-7 cells [1-3]. In these cells, the action of anti-estrogenic compounds has been shown to be mediated by their binding to the ER (predominantly ER-α), altering the dimerization of the receptor and its ability to bind to DNA, or its interactions with accessory proteins [10]. This in turn affects transcription of estrogen responsive genes, some of which are involved in cell cycle progression or regulation of apoptosis including the kinase inhibitor proteins, p21 and p27 which are upregulated and cyclin D1 and Bcl-2 expression which are downregulated by tamoxifen and ICI 182,780 [9, 40, 41]. These reports correlated with our observation that tamoxifen and ICI 182,780 inhibit cell growth and induce cell cycle arrest and high molecular weight fragmentation in MCF-7 cells. The observed inhibition of cell proliferation and cell cycle progression and induction of apoptosis by tamoxifen in ER-negative SUM-159PT and MDA-MB-435 cells however cannot be attributed to regulation through ER-α suggesting that alternative non-ER-α mediated mechanisms of tamoxifen induced apoptosis exist. The data presented in this manuscript suggest that the effects of tamoxifen in the ER-α-negative cell lines are modulated through the ER-β receptor, which is expressed in both the SUM-159PT and MDA-MB-435 cells.

While tamoxifen induces a limited repertoire of caspases in MDA-MB-435 cells (caspase-3 and -9), the DNA cleavage appears to be induced through the classical apoptosome mediated pathway, although there is no evidence of oligonucleosome formation. While tamoxifen induces bax translocation to the mitochondrial membrane and cleavage to t-Bax in SUM-159PT cells, the release of cytochrome c is very limited and there is no activation of caspases-3,-8 and -9. Since tamoxifen does induce significant cell death in SUM-159PT cells, these data suggest that DNA cleavage is mediated by caspase-independent mechanisms. Recent reports show that ER-negative T-leukaemic Jurkat and ovarian A2780 cancer cells are induced to undergo oxidative stress by tamoxifen [21]. It has also been suggested that the multiple changes of tamoxifen on mitochondrial functions, causing changes in respiration, phosphorylation efficiency, and

membrane structure of mitochondria and endoplasmic reticulum may explain the cell death induced by this drug in different cell types and its anti-cancer activity in ER-negative cells [42].

Recent studies by several groups have shown that selective estrogen modulators and anti-estrogens act as antagonists of ER-β whereas tamoxifen is a partial agonist of ER-α [30, 43]. The effects of tamoxifen on the ER-β receptor may explain some or all of the effects mediated by tamoxifen in ER-α-negative cancer cells. The inability of ICI 182,780 to induce apoptosis through ER-β in a manner similar to tamoxifen in ER-negative cancer cells may be due to differences in the affinity of the two drugs for ER-β. The experiments described in this manuscript have all been performed in the presence of estradiol. Both the SUM-159PT and MDA-MB-435 cell lines continue to proliferate in the absence of estradiol, indicating that the induction of apoptosis by tamoxifen is initiated by positive signaling events. In the absence of estradiol, ICI 182,780 induces DNA fragmentation in SUM-159PT cells (Gilmore, unpublished). Thus the presence of estradiol may modulate the sensitivity of breast tumors expressing ER-β to selective estrogen response modulators and pure anti-estrogens. ER-β is expressed in both normal mammary epithelium and in breast cancer and its expression has been correlated with better overall survival rates than for ER-β negative tumors [30-33]. Furthermore ER-β expression is predictive of a positive response to tamoxifen treatment and adjuvant tamoxifen therapy significantly increases survival in patients with ER-β expressing tumors [34]. This may offer an explanation for the subset of patients classified as ER-negative that respond to tamoxifen therapy and suggests that evaluation of ER-β status may identify a new subset of patients who will benefit from tamoxifen therapy. Clearly, determining the mechanisms of tamoxifen mediated apoptosis in ER-β expressing tumors may lead to clinical advances in the diagnosis and treatment of breast cancer patients. Our findings therefore suggest that additional non-ER-α mediated mechanisms exist in ER-negative cell lines.

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FIGURE LEGENDS

Figure 1. Effects of tamoxifen and ICI 182,780 on ER-positive and ER-negative breast cancer cell proliferation. MCF-7 cells were plated at 10,000 cells/well in a 24 well plate. SUM-159PT and MDA-MB-435 cells were plated at 8,000 cells/well. 48 h following plating, cells were treated with ethanol vehicle;  , 10 μM tamoxifen;  or 10 μM ICI 182,780;  for the indicated times. For all treatments total cell number was determined by crystal violet staining. Data expressed as mean ± SEM of four values per time point. Similar result were obtained from at least three other independent experiments. *, P<0.05 (95% confidence level); ethanol control vs treated.

Figure 2. Effects of Tamoxifen and ICI 182,780 on morphology (A) and DNA fragmentation (B) in ER-positive and ER-negative cells. A. Cells grown on Lab-Tek II chamber slides were treated with ethanol vehicle, 10 μM Tamoxifen or 10 μM ICI 182,780, fixed after 72 h and immunostained using an 'In situ Cell Detection Kit" and visualized with DAB (brown nuclear staining). Photographed at 20x magnification. Bar = 50 μ. **B.** MCF-7, SUM-159PT and MDA-MB-435 cells were plated and treated for 48 h with vehicle, 10 μM Tamoxifen or 10 μM ICI 182,780 for 48 h. as described in Materials and Methods. For quantitation of DNA fragmentation, DNA strand breaks were labeled with Br-dUTP and detected using a FITC-conjugated anti-BrdU antibody as described in Materials and Methods. Numbers in upper right corner of each panel indicate the percentage of positive cells after negative subtraction of data generated with vehicle control cells.

Figure 3. Mitochondrial activation after treatment with tamoxifen. A. Cells were plated and treated with 10 μM tamoxifen or 5 μM etopside for 48 h as described in Materials and Methods. Mitochondria and cytosolic fractions, isolated as described in Materials and Methods were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with a

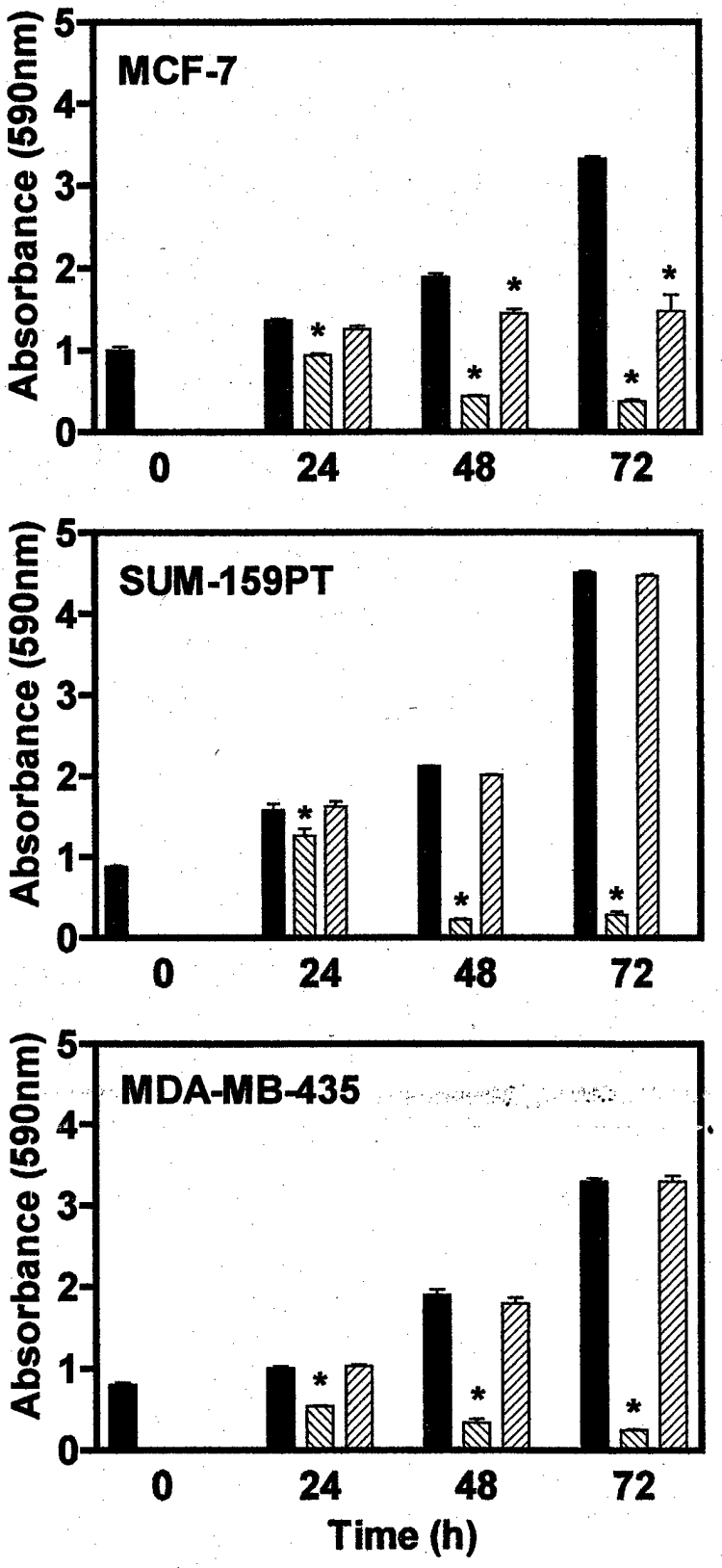
rabbit polyclonal antibody to Bax. **B.** Cells were plated and treated with 10 μM tamoxifen or 5 μM etopside for 48 h as described in Figure 2B. Cytosolic fractions isolated as described in Materials and Methods, were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with a mouse monoclonal antibody to cytochrome c.

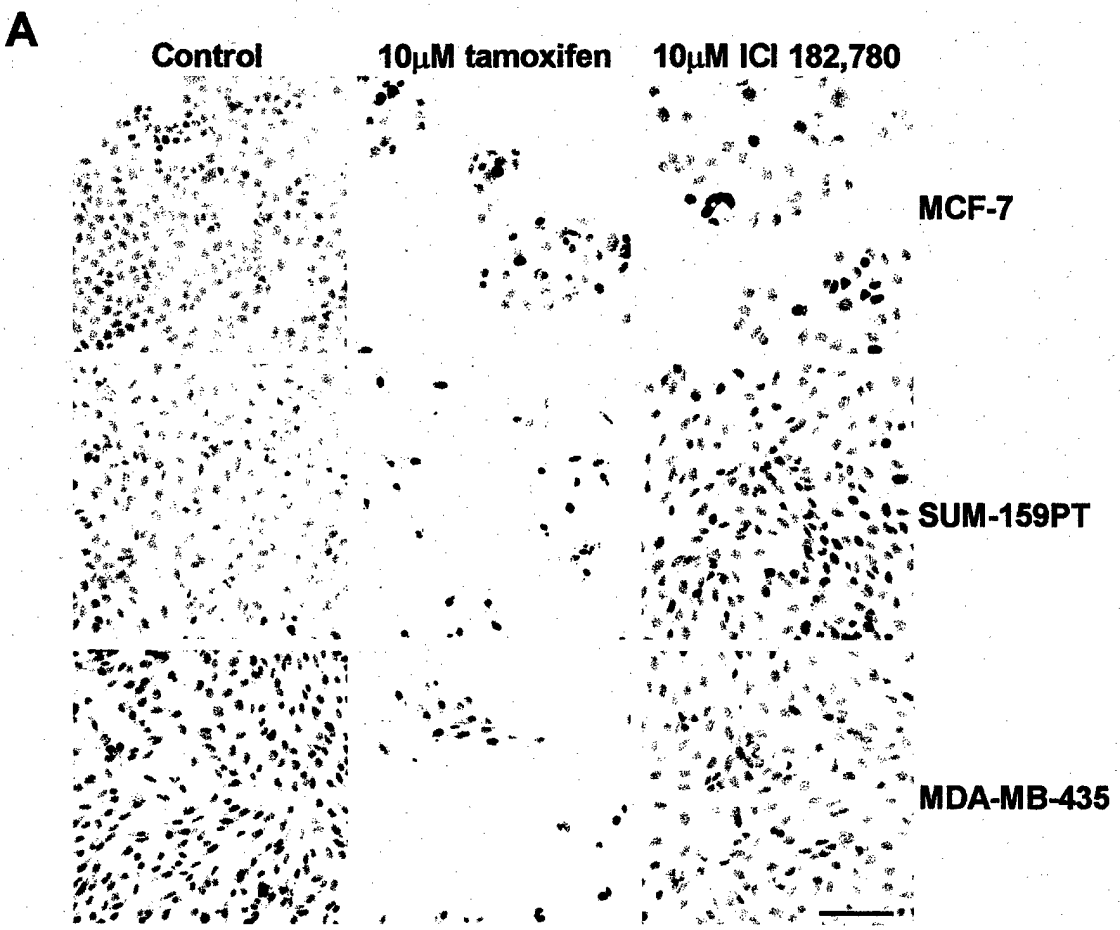
Figure 4. Caspase Activation in ER-negative SUM-159PT (A) and MDA-MB-435 (B) breast cancer cells upon treatment with tamoxifen, ICI 182,780 and etoposide. Cells were plated and treated with ethanol control;  10 μM tamoxifen;  10 μM ICI 182,780;  or 1 μM etoposide;  as described in Materials and Methods for the indicated times. The relative levels of caspase activity in cell lysates were estimated using a fluorescence microplate assay as described in Materials and Methods. Activity levels were determined by comparison of fluorescence emission in the presence or absence of the appropriate substrate inhibitor.
*, $P<0.05$ (95% confidence level); control vs treated.

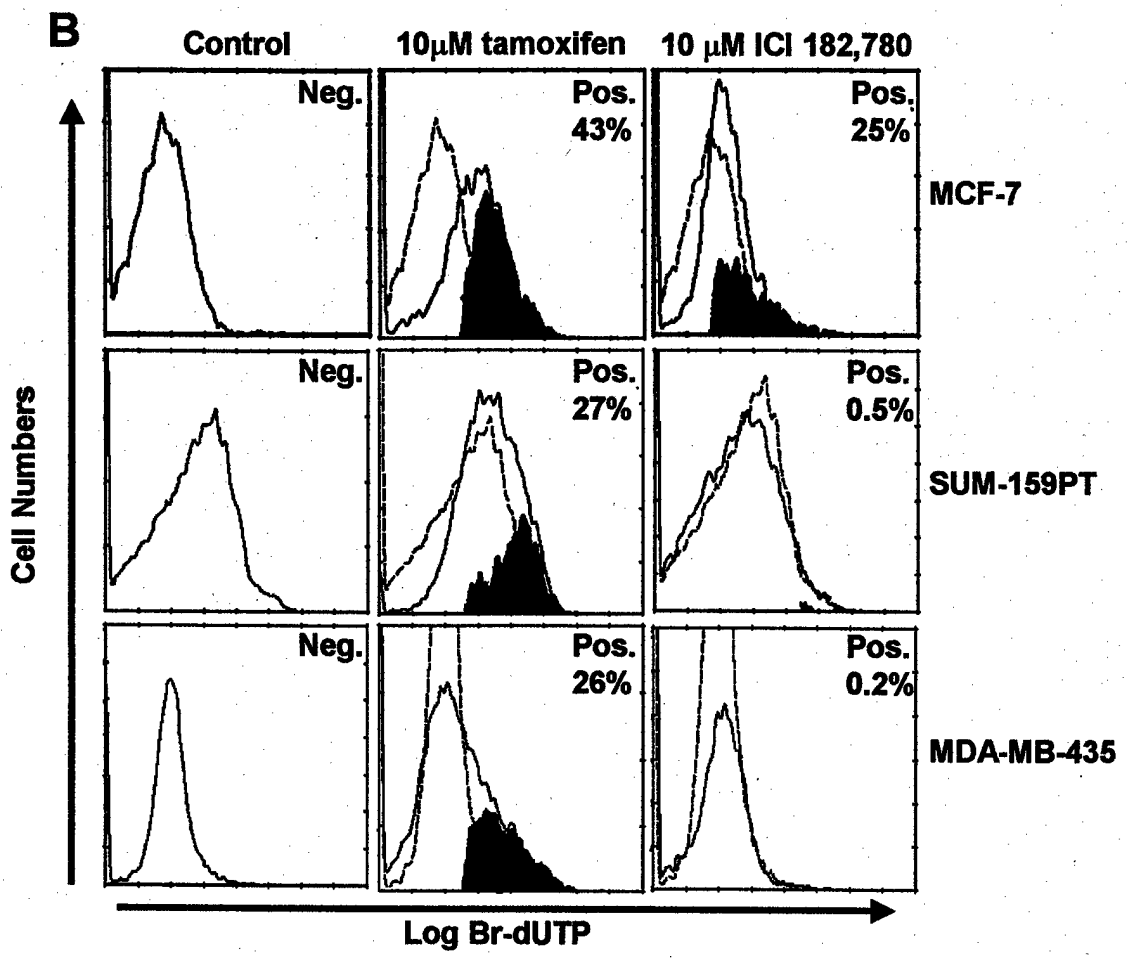
Figure 5. Field inversion gel electrophoresis of ER-negative breast cancer cells upon treatment with tamoxifen, ICI 182, 780 and etoposide. Cells were plated and treated for 24 h as described in Materials and Methods. 2×10^6 cells were embedded in 1% low melting point agarose and proteinase K digestion and electrophoresis was carried out as described in Materials and Methods.

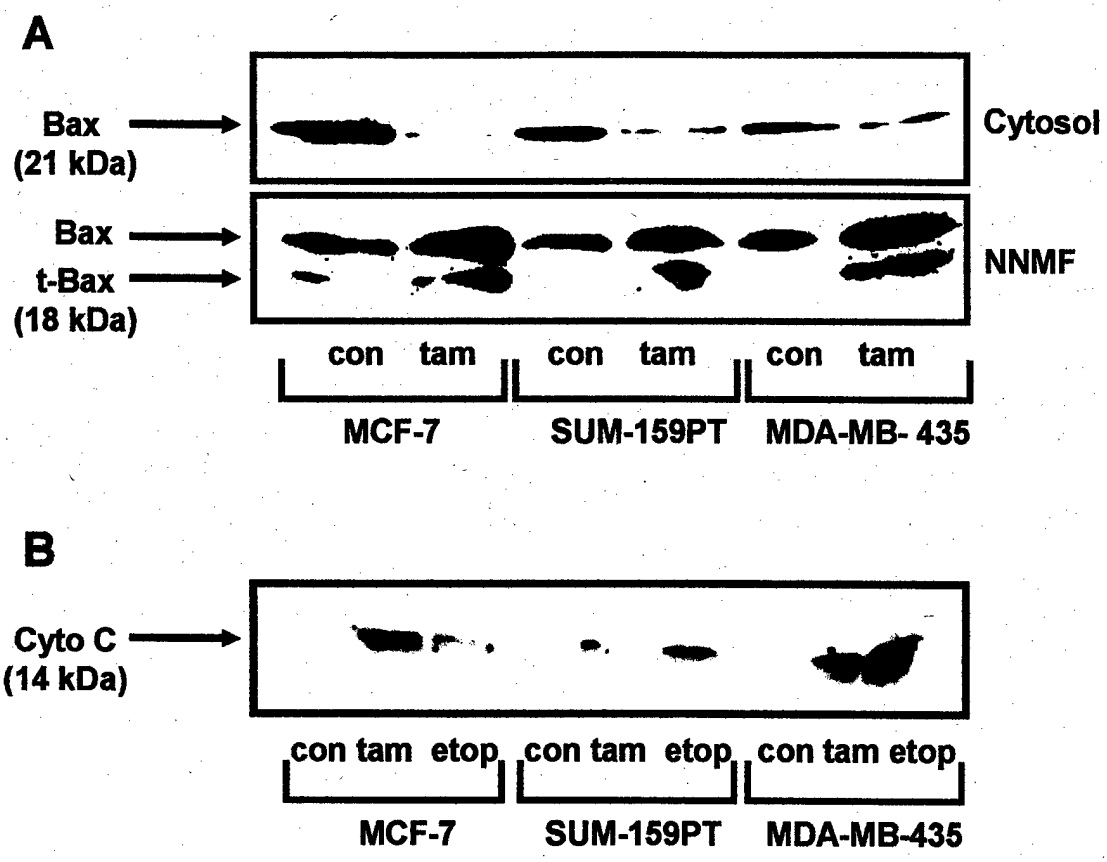
Figure 6: RT-PCR Analysis of ER-α and ER-β in ER-positive and ER-negative breast cancer cell lines. Total RNA was isolated from MCF-7, SUM-159PT and MDA-MB-435 cells and amplified by PCR as described in Materials and Methods.

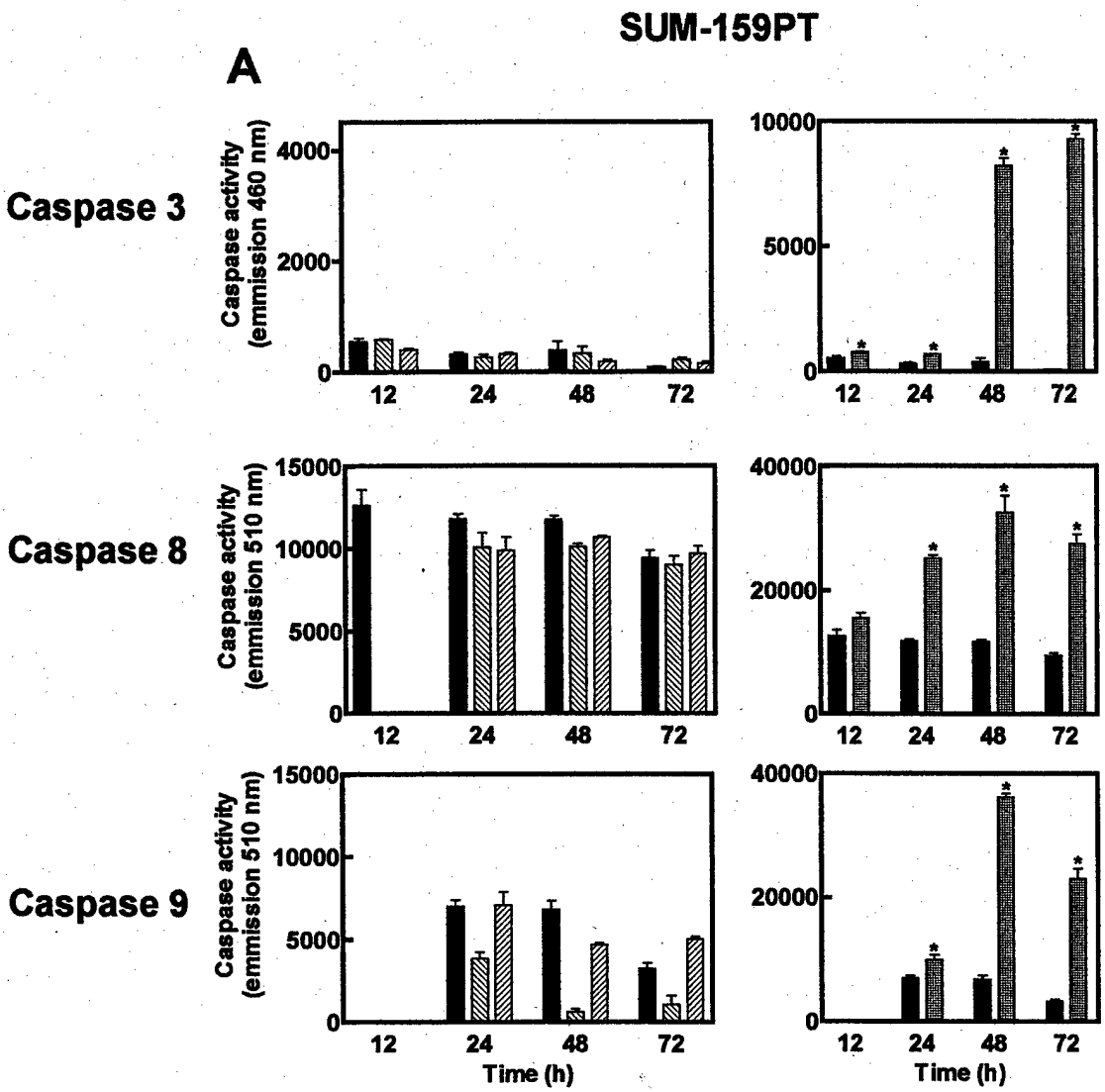
Table 1. Effects of Tamoxifen and ICI 182,780 on Cell Cycle Progression in ER-positive and ER-negative breast cancer cells. Cells plated and treated as described in Materials and Methods for 48 h were ethanol fixed, stained with propidium iodide and analyzed by flow cytometry as described in Materials and Methods.

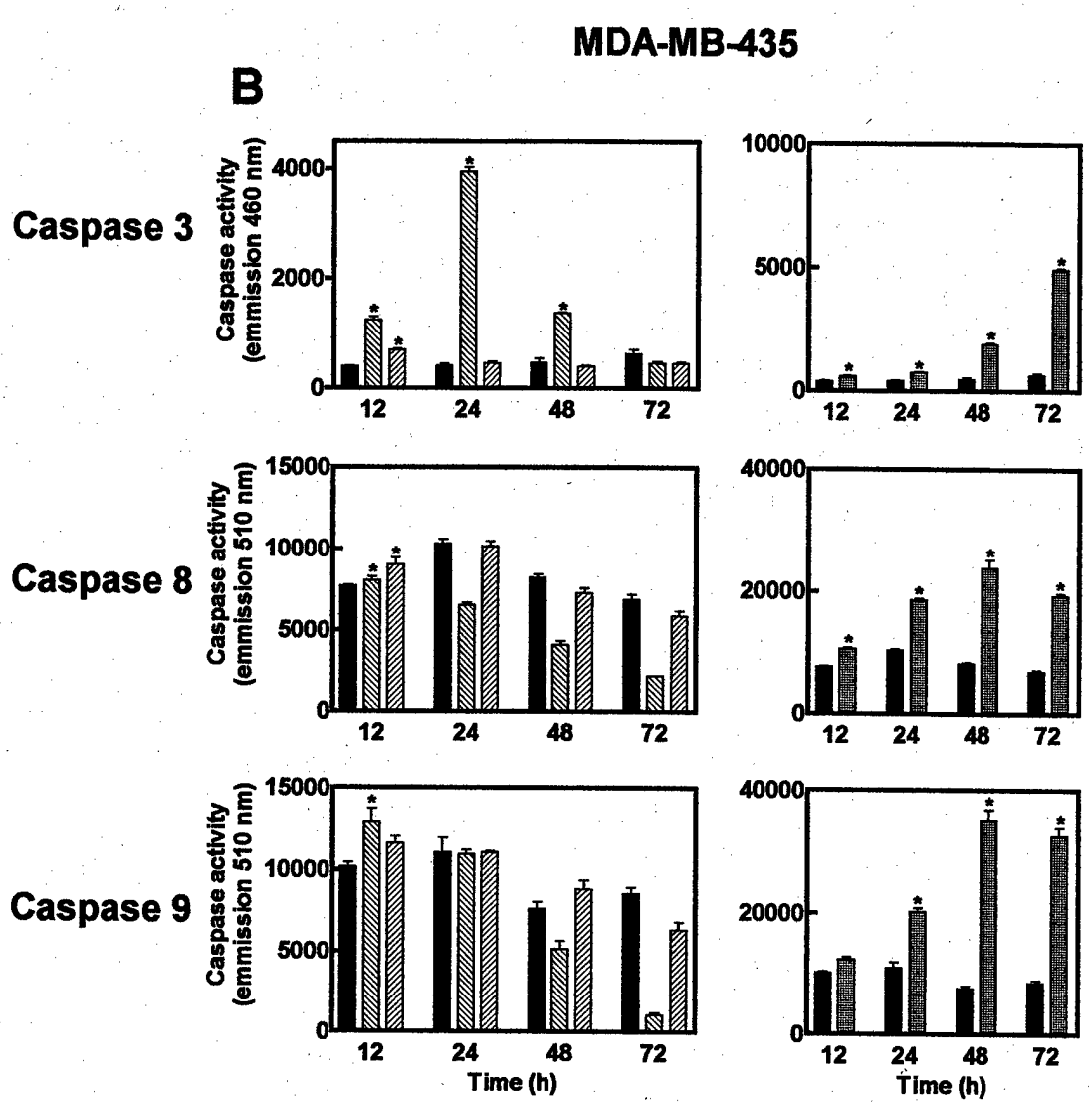

MCF-7
SUM-159PT
MDA-MB-435
Absorbance (590nm)
0
1
2
3
4
5
0
24
48
72
Time (h)
*

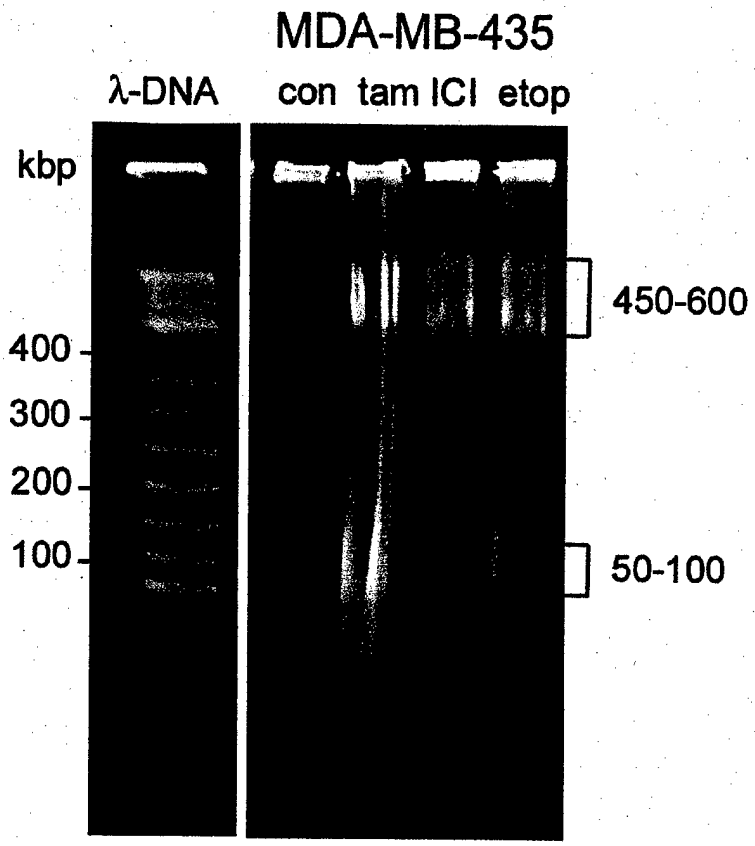

A
Control
10μM tamoxifen
10μM ICI 182,780
MCF-7
SUM-159PT
MDA-MB-435

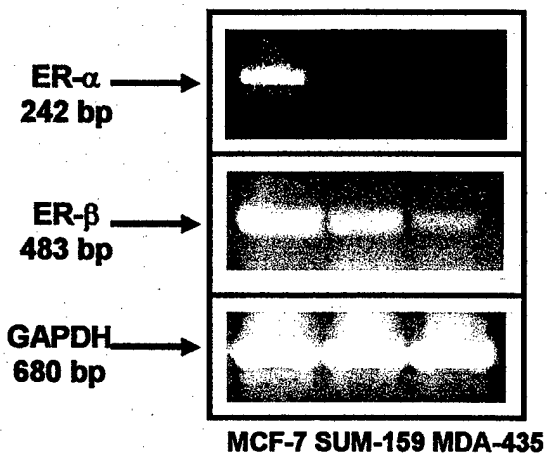

B
Control
10μM tamoxifen
10 μM ICI 182,780
Neg.
Pos.
43%
Pos.
25%
MCF-7
Neg.
Pos.
27%
Pos.
0.5%
SUM-159PT
Neg.
Pos.
26%
Pos.
0.2%
MDA-MB-435
Cell Numbers
Log Br-dUTP


A
Bax
(21 kDa)
Cytosol
Bax
t-Bax
(18 kDa)
NNMF
con tam
con tam
con tam
MCF-7
SUM-159PT
MDA-MB- 435
B
Cyto C
(14 kDa)
con tam etop
con tam etop
con tam etop
MCF-7
SUM-159PT
MDA-MB-435


SUM-159PT
A
Caspase 3
Caspase 8
Caspase 9
Caspase activity (emmission 460 nm)
Caspase activity (emmission 510 nm)
Caspase activity (emmission 510 nm)
4000
2000
0
10000
5000
0
15000
10000
5000
0
40000
20000
0
12
24
48
72
Time (h)


MDA-MB-435
B
Caspase 3
Caspase 8
Caspase 9
Caspase activity (emmission 460 nm)
Caspase activity (emmission 510 nm)
Caspase activity (emmission 510 nm)
4000
2000
0
10000
5000
0
15000
10000
5000
0
40000
20000
0
12
24
48
72
Time (h)


MDA-MB-435
λ-DNA
con tam ICI etop
kbp
400
300
200
100
450-600
50-100


ER-α
242 bp
ER-β
483 bp
GAPDH
680 bp
MCF-7 SUM-159 MDA-435

Percentage of Cells	G_0/G_1			S			G_2M		
	Con	Tam	ICI	Con	Tam	ICI	Con	Tam	ICI
MCF-7	54	87	88	31	5	5	15	8	7
SUM-159PT	55	71	58	33	24	31	12	5	11
MDA-MB-435	42	52	42	46	24	44	12	24	14

Each analysis was performed three times