

# Response of Mouse Breast Cancer Cells to Anastrozole, Tamoxifen, and the Combination

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The murine breast cancer cells (4T1) grown both in female BALB/c mice and in culture were treated with anastrozole (50  $\mu\text{g}/\text{mL}$ ), tamoxifen citrate (5  $\mu\text{g}/\text{mL}$ ), and the combination of the two drugs in order to determine treatment efficacies, toxic potential, and the mechanism of cell death. The *in vivo* treatments were evaluated by monitoring tumor growth, development, and life span. The *in vitro* effects were measured through cell growth kinetics, cell proliferation, mitochondrial membrane potential disruption assay, and light and scanning electron microscopy. All drug treatments extended the mean life span of the 4T1-inoculated tumor-bearing mice; however, only tamoxifen and combination treatments statistically increased the life span when compared to untreated mice. Although the most drug inhibitory effect on cell multiplication was observed in the combination treatment, both anastrozole and tamoxifen individually inhibited cell proliferation significantly at most time periods in this mouse breast cancer cell line. The mitochondrial membrane potential disruption assay demonstrated significant increase in the percent of cells undergoing apoptosis in all treatment groups. However, the combination treatment was the most effective in inducing cell death via apoptosis. Light and scanning electron microscopy of the treated cells revealed characteristics such as rounding, clumping, and shrinkage of the cells as well as formation of cell surface blebbing and apoptotic bodies suggestive of cell death via apoptotic pathway.

## INTRODUCTION

For the past twenty years, tamoxifen has been the drug of choice for the treatment of advanced-stage estrogen receptor positive (ER-positive) breast cancers [1, 2]. This antibrast cancer drug is often successful in limiting breast cancer growth initially, but continued use of the drug was known to stimulate disease progression [3, 4]. Because drug treatment with tamoxifen is not ideal, other hormonal chemotherapies have been developed. Second-generation treatments were developed but were overshadowed by the rapid success and development of the third-generation aromatase inhibitors such as anastrozole, letrozole, and vorozole [5]. The most popular of the aromatase inhibitors is anastrozole, or Arimidex, which is used to treat ER-positive breast cancers in postmenopausal women. Anastrozole binds to aromatase eliminating its estradiol synthesizing function, resulting in a drop in the circulating level of estrogen [6, 7]. In 1995, anastrozole was originally approved by the Food and Drug Administration (FDA) as the first nonsteroidal aromatase inhibitor for the treatment of tamoxifen-resistant ER-positive breast cancer [8, 9]. Later, in 2000, anastrozole was approved for first-line treatment of advanced ER-positive breast cancer by the FDA [10, 11]. In June 2002, the preliminary results of a massive anastrozole study comparing its effectiveness alone and in combination with tamoxifen (ATAC) found that anastrozole alone was

statistically more effective than either tamoxifen alone or in combination with anastrozole in the treatment of breast cancer in postmenopausal women [12]. In recent studies, anastrozole was shown to shrink tumor size allowing previously inoperable breast cancers to become operable [13].

Studies aimed at comparing the efficacies of anastrozole and tamoxifen in various breast cancer treatment settings and stages have produced conflicting results. A multicenter randomized trial revealed that anastrozole was more effective than tamoxifen in slowing breast cancer tumor progression in women diagnosed with advanced-stage breast cancer [10]. In contrast, a similar multicenter randomized trial conducted by Bonnetterre et al [14] reported no significant difference in tumor progression between women treated with anastrozole and those treated with tamoxifen. Furthermore, information on the effectiveness of anastrozole, tamoxifen, and their combination on nonmammalian breast cancer cells both in *in vivo* and *in vitro* settings is limited. Consequently, future studies evaluating the efficacies of tamoxifen, anastrozole, and the combination in various mammalian systems involving different breast cancer cell types are needed in order to evaluate the best strategy for the treatment of breast cancers. To the best of our knowledge, no reports on the effects of treatments with anastrozole, tamoxifen, and the combination on this mouse breast cancer cell line (4T1) have been published.

The 4T1 cells grow as adherent epithelial type in vitro, and are characterized as murine mammary carcinoma cells (American Type Culture Collection (ATCC) catalogue no. CRL-2539, 2004). When injected into BALB/c mice, 4T1 cells rapidly multiply resulting in highly metastatic tumors. Because these tumors closely imitate human breast cancer, the 4T1 cell line serves as an animal model for stage IV breast cancer [15]. In this investigation, we evaluated the effectiveness of anastrozole, tamoxifen, and the combination on 4T1 cells' ability to induce tumors in BALB/c female mice. Various parameters such as tumor induction capability, tumor mass, and mean life span were recorded to determine the drugs' efficacy on 4T1 cells. Additionally, the drugs' effects on 4T1 cell growth kinetics, proliferation, and morphology were investigated. In order to determine the mechanisms of the death of 4T1 cells exposed to individual and combination drug treatments, a mitochondrial membrane potential disruption assay and a scanning electron microscopy (SEM) investigation were performed.

## MATERIALS AND METHODS

### Cell maintenance

In vivo and in vitro studies were performed utilizing the 4T1 cell line. This cell line was supplied to this laboratory by Dr. Robert Kurt (Lafayette College, Easton, Pa). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, Md) supplemented with 10% fetal calf serum (Sigma Aldrich, St Louis, Mo) (DME-10) and kept in a 37°C humidified incubator with 7.5% CO<sub>2</sub> in air. The 4T1 cells used in the tumor development study were freshly harvested by Dr. Robert Kurt from 4T1-induced tumors in BALB/c mice.

### Mouse maintenance

Three-to-four-week-old female BALB/c mice were purchased from Ace Animals (Boyerstown, Pa). The mice were housed four or five to a cage, fed ad libitum, and observed daily. The cages were kept in a climate-controlled warm animal suite and cleaned weekly. Approval for this study was attained from the Lafayette College Institutional Animal Care and Use Committee (IACUC) and the mice were handled and euthanized according to its guidelines.

### Drug preparation

Tamoxifen citrate ((Z)-1-(p-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene) was purchased from Sigma Aldrich (St. Louis, Mo). The tamoxifen citrate powder was dissolved in a phosphate buffered saline (PBS) solution to a concentration of 1000 µg/mL and stored at -20°C; this served as a stock solution. The 4T1 cells utilized in the in vitro studies were treated with a 5 µg/mL concentration of tamoxifen citrate. In the in vivo study, each mouse was injected subcutaneously with 5 µg of tamoxifen citrate in 0.1 mL of PBS. Injection protocols are provided in the "tumor development study" section.

Arimidex, 1 mg anastrozole tablets, manufactured by Astra Zeneca (London, England) were obtained through the Bailey Health Center (Lafayette College, Easton, Pa) from Bell's Apothecary (Easton, Pa). The tablets were dissolved in PBS to a final concentration of 500 µg/mL stock solution and stored at -20°C. Cells for in vitro studies were treated with 30 µg/mL or 50 µg/mL of anastrozole, while mice in the in vivo study were injected as described above with 50 µg of anastrozole per mouse daily during the treatment period. The combination of anastrozole (50 µg/mL) and tamoxifen (5 µg/mL) was used for both in vitro and in vivo investigations.

Camptothecin, a known inducer of apoptosis [16], was purchased in powder form from Sigma Aldrich (St. Louis, Mo) and was dissolved in PBS to a final concentration of 1000 µg/mL. The agent was used as a positive control in the mitochondrial membrane potential disruption study (3.5 µg/mL).

### Tumor development study

Seven-week-old female BALB/c mice were injected subcutaneously with 10<sup>5</sup> 4T1 cells suspended in 0.1 mL of PBS on the upper portion of the right hind thigh on day zero of the study. The individual or combination regimens of each drug dissolved or suspended in 0.1 mL PBS were injected subcutaneously on the inside of either thigh every 48 hours with a 25 gauge syringe. Each mouse received 14 doses of its designated drug over a 28 day period. Mice in the untreated group were similarly given 14 doses of 0.1 mL of PBS.

The day of tumor induction was recorded. Tumor volume (tumor volume = length × width<sup>2</sup> × 0.5) was recorded twice per week following the equation utilized by Kotoh et al and Ruddy and Majumdar in similar tumor development studies [17, 18]. The mice were examined every other day for maladies including rough coat appearance, discoloration of skin, and swollen abdomen. Mice were sacrificed approximately 1–2 days prior to their natural death by cervical dislocation and life span was recorded. After euthanization, the mice were dissected. The primary tumor, liver, and spleen were removed, studied, and weighed.

### Cell kinetics study

Fifty thousand 4T1 cells were seeded in each well of a six-well plate in 4 mL of DME-10 and treated with tamoxifen citrate, anastrozole, and the combination of the two drugs. The 4T1 cells were harvested using a 0.25% buffered trypsin solution (Gibco BRL, Rockville, Md), and viable cells were counted using the trypan blue exclusion method at 24-hour intervals for five days.

### Cell proliferation study

Ten thousand exponentially growing 4T1 cells were added to each well of a 96-well plate as suggested by the Quick Cell Proliferation Assay Protocol obtained from Biovision (Quick Cell Proliferation Assay Kit, Mountain

TABLE 1. Summarized results of the tumor development study. Female BALB/c mice were inoculated with 10 000 4T1 cells subcutaneously in the right hind thigh and treated with anastrozole, tamoxifen, the combination, or the untreated phosphate buffered solution.

	Mean tumor detection day $\pm$ SEM	Mean life span $\pm$ SEM	Mean tumor mass at death $\pm$ SEM
Untreated	10.8 $\pm$ 1.1	24.8 $\pm$ 1.7	0.841 $\pm$ 0.325
Anastrozole	11 $\pm$ 0.7	26.9 $\pm$ 1.6	1.252 $\pm$ 0.308
Tamoxifen	9.5 $\pm$ 0.7	31 $\pm$ 1.1* $\diamond$	1.840 $\pm$ 0.173*
Anastrozole + Tamoxifen	10.5 $\pm$ 0.7	29 $\pm$ 1.6*	1.413 $\pm$ 0.215

\*Statistical difference from the untreated control group ( $P \leq .05$ ), as determined by the Student  $t$  test.

$\diamond$ Statistical difference from the anastrozole-treated group ( $P \leq .05$ ), as determined by the Student  $t$  test.

View, Calif). Anastrozole, tamoxifen, or the combination of the two drugs was added to the cells in each well. A blank was prepared by adding only culture media to a row of wells. Twenty-four hours later, each well was supplied with 10  $\mu$ L of a mixture of the tetrazolium salt WST-1 and an electrocoupling solution. The plate was incubated for three hours in a 37°C humidified incubator with 7.5% CO<sub>2</sub> in air. Mitochondrial dehydrogenases found in viable cells converted WST-1 to a formazan dye during this incubation period. Dye formation, indicative of cell viability, was quantified by measuring absorbency at 450 nm with an EL312E Automated Microplate Reader (Bio-Tek instruments, Winooski, Vt). After 24 hours of treatment, the cells were studied under an inverted Olympus microscope (Melville, NY) at 100 $\times$  magnification and photographed digitally using a MX-700 fujifilm digital camera (Tokyo, Japan).

#### Mitochondrial membrane potential ( $\Delta\psi$ ) disruption assay

The 4T1 cells were grown on square sterile coverslips in 60  $\times$  15 mm tissue culture dishes with 2 mL of DME-10. After 24 hours the cells were treated with tamoxifen citrate, anastrozole, or a combination of tamoxifen citrate and anastrozole in varied concentrations as specified above for 24, 48, and 72 hours. Camptothecin (3.5  $\mu$ g/mL), a known apoptotic inducer, was used as a positive control [16]. Following the Trevigen protocol, using the DePsipher Mitochondrial Membrane Potential Disruption Kit (Trevigen, Inc, Gaithersburg, Md), the incubating solution was prepared which contained 1X reaction buffer, stabilizing solution, and the DePsipher solution. The adherent cells on coverslips were incubated for 30 minutes in the above solution in a humidified incubator at 37°C in 7.5% CO<sub>2</sub> in air. The slides were examined under a fluorescence microscope (Bausch and Lomb, Rochester, NY) with a dual filter set of FITC and rhodamine for detection of mitochondrial membrane potential disruption. The normal membrane potential of mitochondria was indicated when the DePsipher lipophilic cation (5,5',6,6',tetrachloro-1,1',3,3',tetraethylbenzimidazolyl carbocyanin) aggregated on the membrane forming a red-orange fluorescent

compound. However, if the membrane polarization is disrupted, the cation cannot access the mitochondrial transmembrane space and the cells remain green. This is an early indication of apoptosis. Approximately 300 cells per treatment and time interval were examined. Photographs were taken using an Olympus C-35 camera (Tokyo, Japan) with ISO 1600 speed film utilizing a 30-second time exposure.

#### Scanning electron microscopy

The 4T1 cells were grown on sterile circular coverslips in small culture dishes in 3 mL of DME-10 with either tamoxifen citrate, anastrozole, or the combination of the two drugs. After 48 hours of drug treatment, the cells were fixed in 2% glutaraldehyde, post fixed in a 1% osmium tetroxide solution, dehydrated in an ascending series of ethanol, and critically point dried (Polaron E3000, Mountain View, Calif). The cells were sputter-coated with gold palladium using a Cressington 108 auto sputter coater (Cranberry Township, Pa) and examined with an ISI Super III A scanning electron microscope. Photographs were taken with B52 and B55 Polaroid pan films as well as the Printerface computer program.

#### Statistical analysis

Statistical analyses pertaining to toxicological experiments were performed according to the statistical methods recommended by Gad and Weil [19]. These included Bartlett's homogeneity of variance, unpaired Student's  $t$  test, F test, Tukey's test, chi-square test, and Fisher's exact test [19, 20]. Microsoft Excel was used to compile data and to create corresponding graphs and tables.

## RESULTS

### Tumor development study

Table 1 summarizes the results of the tumor development study. Mice were sacrificed approximately 2–3 days prior to natural death by cervical dislocation. Matting and thinning of fur, discoloration of skin, substantial tumor size causing paralysis, large swollen abdomens, frailty or thinning, and overall lethargy were the characteristics used as indicators of near death. The mean life span

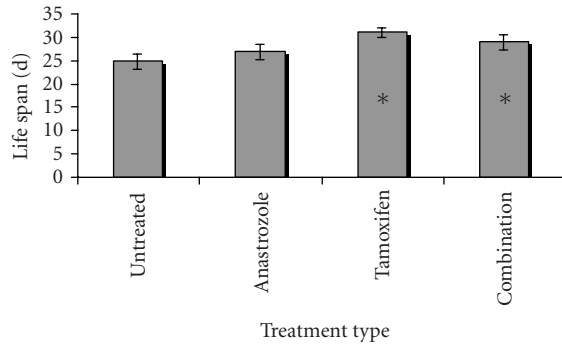


FIGURE 1. Mean life spans of 4T1-inoculated mice. Stars indicate statistical difference from the untreated group ( $P \leq .05$ ), as determined by the Student  $t$  test. Bars represent  $\pm$  SEM.

of each treatment group was calculated. Utilizing an unpaired unequal variance Student  $t$  test, the mean life spans of the tamoxifen-treated mice were found to be significantly longer than the mean life spans of the mice in the anastrozole treated and untreated groups (see Figure 1). The combination treatment also extended the life spans of the mice which were found to be statistically different from the untreated group ( $P < .05$ ) but not from either the anastrozole- or tamoxifen-treated groups (see Table 1). Tamoxifen appeared to be most effective in the trial. Tumor volume was measured throughout the experiment but no statistical significance was found on any day across the treatment groups. After death the tumors were removed and studied. Mean tumor mass at death was calculated and the average tumor mass of tamoxifen-treated mice was found to be significantly larger than that of the untreated group by the Student  $t$  test (see Table 1). Those mice with both swollen abdomens and discolored skin, sacrificed early due to their very ill appearance, had smaller tumors. Upon dissection, the abdomens of these mice were found to be filled with a dark brown fluid. When the fluid was removed from its source and injected into BALB/c female mice, 100% tumor formation occurred at the site of injection within 14 days.

Because of the high metastatic characteristic of 4T1 cells, the spleens and livers of 4T1-inoculated mice were also examined post mortem for evidence of secondary cancer development. Spleen enlargement was observed in 4T1-inoculated mice that did not receive the drug (see Figure 2). In the untreated group, the spleen mass was as much as ten times larger than that of a cancer-free mouse. Although there was no statistical difference in the averages of spleen size at death across the treatment groups, mice sacrificed from each group on day 28 of the experiment exhibited clear size and mass differences. The largest spleen size and mass were detected in the 4T1-inoculated mouse that received no drug treatment. The spleen size and mass increased over the remaining treatments of

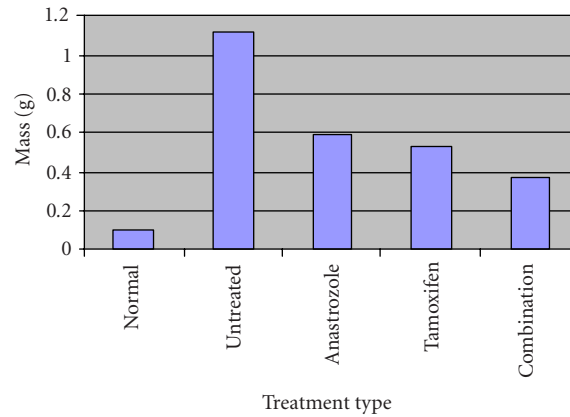


FIGURE 2. Spleen masses of female BALB/c mice on day 28 of the experiment. All mice except the normal (negative control) were inoculated with 4T1 cells 28 days prior to spleen removal and treated with either PBS, anastrozole, tamoxifen, or the combination.

anastrozole-, tamoxifen-, and combination-treated mice, respectively. Cancerous nodules often appeared in the livers of 4T1-infected mice although no significant variation in mean liver mass at death was found across the treatment groups. Furthermore, the mean tumor detection day across the treatment groups revealed no statistical difference (see Table 1).

### Cell multiplication study

Cell growth inhibition of 4T1 in presence of anastrozole was in general concentration-dependent. The 50  $\mu\text{g}/\text{mL}$  anastrozole treatment statistically reduced viable cell number compared to the untreated control at all time periods, while the 30  $\mu\text{g}/\text{mL}$  anastrozole treatment differed significantly from the untreated cells only at 96 and 120 hours (see Figure 3). When 30  $\mu\text{g}/\text{mL}$  and 50  $\mu\text{g}/\text{mL}$  of anastrozole treatments were compared, a statistical difference in the number of viable 4T1 cells was detected at the 72-hour time period.

Both the tamoxifen (5  $\mu\text{g}/\text{mL}$ ) and the combination treatment (anastrozole 50  $\mu\text{g}/\text{mL}$  and tamoxifen 5  $\mu\text{g}/\text{mL}$ ) inhibited 4T1 cell growth (see Figure 4). The number of viable cells in the tamoxifen- and combination-treatment groups was statistically fewer than in the untreated cells at 48, 72, 96, and 120 hour time periods. The combination treatment was found to be a more effective inhibitor of 4T1 viability than the tamoxifen treatment at all time periods. At all time periods, the number of viable 4T1 cells treated with the combination was less than the number of cells used in the initiation of the study. When compared to anastrozole (50  $\mu\text{g}/\text{mL}$ ), the combination treatment was found to exert a greater inhibitory effect on cell multiplication; anastrozole alone (50  $\mu\text{g}/\text{mL}$ ), however, was more effective than tamoxifen in reducing 4T1 cell viability following 24 hours of drug treatment.

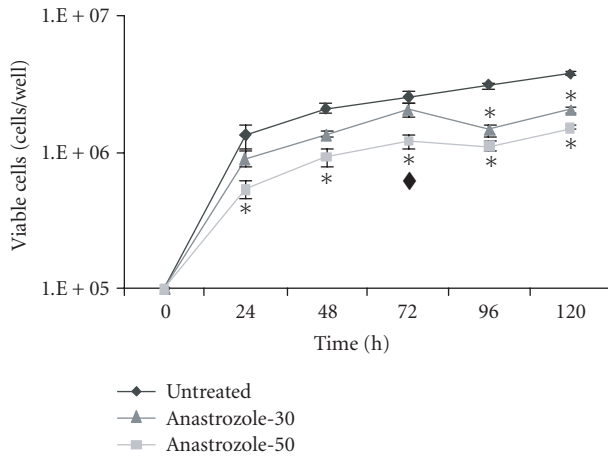


FIGURE 3. Cell growth kinetics of untreated and anastrozole-treated 4T1 (30 or 50  $\mu\text{g}/\text{mL}$ ). Stars indicate a statistical difference in number of viable cells from the untreated groups ( $P \leq .05$ ), as determined by the Student *t* test. A diamond indicates a statistical difference between the two anastrozole concentration groups. Bars represent  $\pm$  SEM.

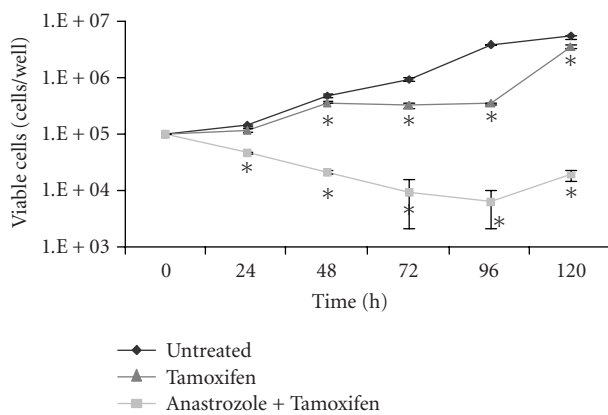


FIGURE 4. Growth kinetics of untreated, tamoxifen-treated (5  $\mu\text{g}/\text{mL}$ ), and combination-treated (anastrozole 50  $\mu\text{g}/\text{mL}$  and tamoxifen 5  $\mu\text{g}/\text{mL}$ ) 4T1 cells. Combination treatment drastically reduced cell number throughout the study period. Stars indicate statistical difference in number of viable cells from the untreated groups ( $P \leq .05$ ). Bars represent  $\pm$  SEM.

### Cell proliferation

In order to determine 4T1 cell viability following 24 hours of drug treatment, a WST-1 solution was added to each well and converted to a quantifiable yellow dye by mitochondrial dehydrogenases present in viable cells, thus serving as an indirect gauge of 4T1 viability. (See "materials and methods.") A microplate reader was used to measure the absorbency of the solution in each well. Statistical analysis of the absorbencies revealed a decrease in 4T1 cell viability following 24 hour anastrozole and combination drug treatments when compared to untreated cells. No

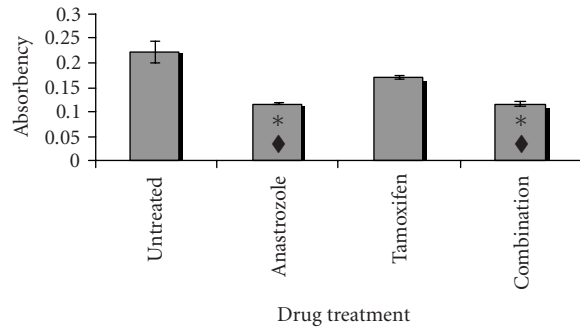


FIGURE 5. Cell proliferation assay results utilizing absorbency (450 nm) as a measure of viability in cells untreated or treated with anastrozole (50  $\mu\text{g}/\text{mL}$ ), tamoxifen (5  $\mu\text{g}/\text{mL}$ ), or the combination (anastrozole and tamoxifen). Stars indicate a statistical difference from the untreated groups ( $P \leq .05$ ). Diamonds indicate a statistical difference from the tamoxifen group ( $P \leq .05$ ). Bars represent  $\pm$  SEM.

statistical difference was detected between the anastrozole and combination treatments (see Figure 5). Additionally, both anastrozole- and combination-treatment groups had significantly less viable cells than the tamoxifen-treated group.

### Light microscopy

Untreated samples exhibited the cell's normal morphology (see Figure 6a). The adherent cells are either rounded or are stretched to an elongated shape by the presence of long cytoplasmic processes called lamellipodia. In contrast, most cells in the drug-treated groups appeared rounded and irregular in shape and exhibited clumping as well as destruction of cell membrane, cell lysis, and cell fragmentation (see Figures 6b, 6c, and 6d). In addition to the heterogeneous appearance, there was also a decrease in cell number in all treatment groups; however, this effect was more pronounced in the tamoxifen and combination groups.

### Mitochondrial membrane potential disruption

In most cases treated cells appeared rounded and green under the fluorescence microscope due to the mitochondrial membrane disruption in the DePispher assay pointing to an early sign of apoptosis. In contrast, 68%–72% of untreated cells showed red-orange coloration during the 72 hour study period indicating nondisruption of the mitochondrial membrane (see Figure 7). Chi-square analysis demonstrated significant differences among the untreated and treated groups, signifying that these two drugs alone and in combination elicited a higher incidence of apoptosis. As expected, the combination treatment was found to be the most potent in inducing cell death possibly via apoptosis which generally paralleled camptothecin treatment. The incidence of cell death observed between the combination and tamoxifen was

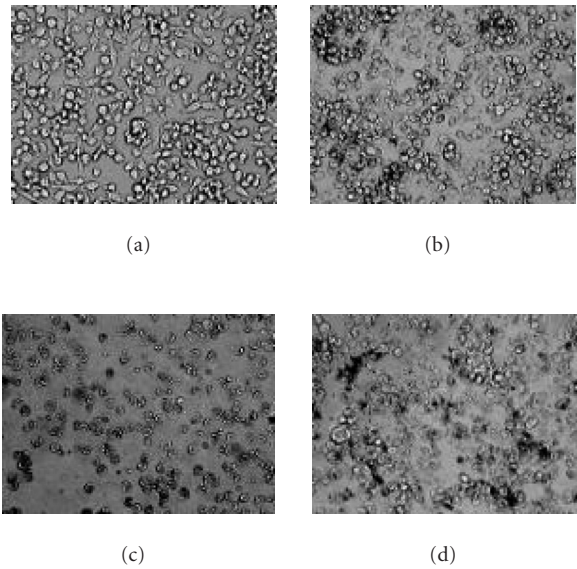


FIGURE 6. Light microscopy images of 4T1 cells (a) untreated or treated for 24 hours with (b) anastrozole (50  $\mu\text{g}/\text{mL}$ ), (c) tamoxifen citrate (5  $\mu\text{g}/\text{mL}$ ), or (d) the combination of the two drugs. Untreated 4T1 cells are either rounded or flattened. Drug-treated cells appear mostly rounded in shape, clumped, and disrupted ( $\times 100$ ).

statistically different at 72 hours. Tamoxifen compared to anastrozole appeared more effective in inducing death in 4T1 mouse breast cancer cells (see Figure 7).

### Scanning electron microscopy

The two drugs alone or in combination induced surface ultrastructural changes in 4T1 cells (see Figure 8). Untreated 4T1 cells were both spherical and flattened with long cytoplasmic processes (see Figure 8a). The untreated groups exhibited certain frequencies of clumping normally after 48 hours; this is depicted at higher magnification in Figure 8b. The cultures exposed to anastrozole for 24 hours showed rounding and clumping as well as thinning of lamellipodia (see Figure 8c). Various surface abnormalities including apoptotic bodies and blebs as well as lysed cells were discerned at higher magnification (see Figure 8d).

Significantly fewer cells and more cell clumping were observed in the tamoxifen-treated group as compared to untreated cells viewed at the same magnification. The tamoxifen-treated cells were irregular in shape with little or no lamellipodia. Formation of apoptotic bodies, membrane blebbing, and holes in the cell membrane was evident (see Figure 8e). Exposure to the combination of anastrozole and tamoxifen resulted in the most drastic changes in cell numbers and ultrastructure (see Figure 8f). Membrane blebs, holes, and apoptotic bodies as well as lysed and irregularly shaped cell clumps of various sizes were detected.

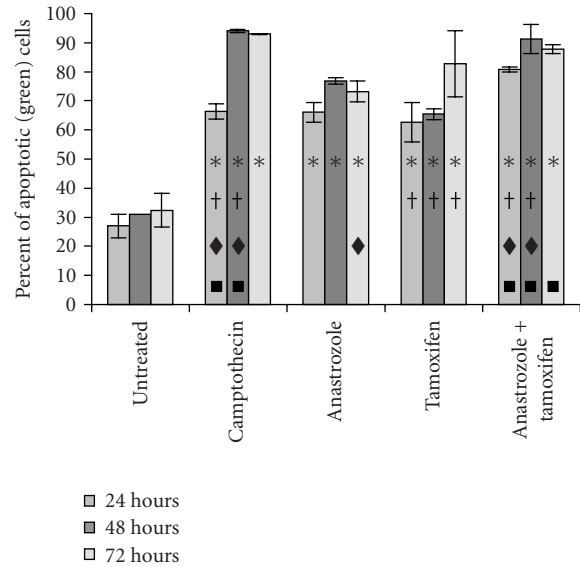


FIGURE 7. Incidence of apoptotic 4T1 cells as detected through mitochondrial membrane potential disruption assay. Stars indicate statistical difference (chi-square) from the untreated group ( $P < .05$ ). Statistical difference among the time periods is indicated by crosses. Diamonds demonstrate statistical difference between tamoxifen (5  $\mu\text{g}/\text{mL}$ ) and anastrozole (50  $\mu\text{g}/\text{mL}$ ), camptothecin (3.5  $\mu\text{g}/\text{mL}$ ), and the combination (50 + 5  $\mu\text{g}/\text{mL}$ ) whereas squares indicate statistical difference among anastrozole, camptothecin, and the combination. Bars indicate  $\pm 1$  SEM.

### DISCUSSION

Since the 1970, tamoxifen has been used to treat hormone-sensitive breast cancers. However, studies have shown that prolonged treatment with tamoxifen may yield tamoxifen-resistant breast cancer [3]. In 1995, anastrozole, a third-generation nonsteroidal inhibitor, was introduced and later approved to treat both early- and advanced-stage ER-positive breast cancers [10, 21]. Still, efficacy data comparing the two drugs on various breast cancer types are incomplete [22]. This study was initiated to investigate the effects of the two drugs alone and in combination on mouse breast cancer cells (4T1 cell line) in inducing tumor in BALB/c female mice and the drugs' role in 4T1 cell growth kinetics, proliferation, and morphology and to decipher the mechanisms of drug-induced cell death.

This study has shown that treatment with tamoxifen significantly increased the life spans of 4T1-inoculated mice when compared to anastrozole-treated or-untreated mice. Similarly, the combination treatment with anastrozole and tamoxifen also lengthened the lives of mice when compared to untreated mice. There was no significant difference between the tamoxifen and combination groups even though the mice in the combination groups received full doses of both drugs. Therefore, anastrozole did not appear to be effective alone or in combination in this in vivo mouse study.

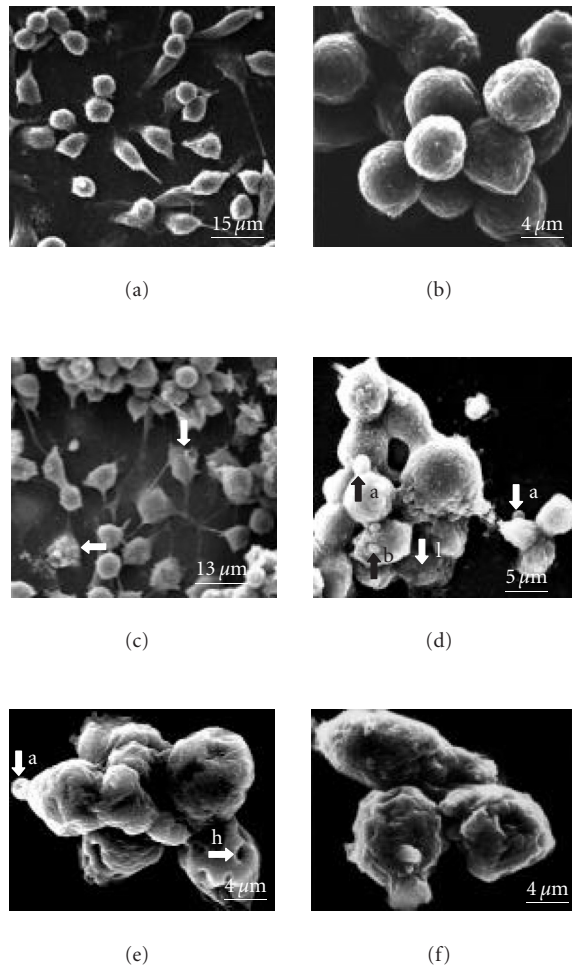


FIGURE 8. Scanning electron micrographs of untreated and 24-hour-treated 4T1 cells with anastrozole (50  $\mu\text{g}/\text{mL}$ ), tamoxifen citrate (5  $\mu\text{g}/\text{mL}$ ), and the combination of the two drugs (50  $\mu\text{g}/\text{mL}$  and 5  $\mu\text{g}/\text{mL}$ ). (a) Characteristics of untreated 24 hour culture showing rounded and flattened cells. (b) Normal surface characteristics of untreated clumped cells shown at 48 hour. (c) Twenty-four-hour anastrozole-treated 4T1 cells showing various surface abnormalities (arrows). (d) Enlarged view of 24-hour anastrozole-treated 4T1 cells depicting apoptotic bodies (a), blebs (b), and lysed cells (l). (e) Tamoxifen-treated cells at 24 hour depicting irregularly shaped cells with apoptotic bodies (a) and holes (h). (f) Anastrozole- and tamoxifen-treated cells. The irregularly shaped cells depict many surface ultrastructural changes including blebs and membrane holes.

Preliminary reports from large-scale studies like ATAC reported that anastrozole's efficacy in humans was superior to that of tamoxifen in the adjuvant setting [12]. A study conducted by Lu et al [23] found that treatment with letrozole, another aromatase inhibitor, was more effective in suppressing human tumor growth in nude mice than the tamoxifen treatment or the combination treatment of letrozole and tamoxifen. In a similar study conducted by Long et al [24], letrozole again demonstrated

superior efficacy when compared to tamoxifen in terms of time for tumor progression in mice. However, the female BALB/c mice used in our study contained both ovaries unlike the mice utilized in the studies conducted by Lu et al [23] and Long et al [24]. Since ovaries are the main site of estrogen production in premenopausal women, it is reasonable to infer that estrogen levels in these mice were significantly higher than those in ovariectomized mice. Premenopausal women have estrogen levels of approximately 379  $\text{pcg}/\text{mL}$ , while postmenopausal women have estrogen concentrations around 83  $\text{pcg}/\text{mL}$  [25]. Therefore, even if anastrozole is blocking estrogen production by inhibiting aromatase, significant levels of estrogen would still be present and thus estrogen-stimulated tumor progression could proceed.

Tamoxifen works by binding directly to the estrogen receptor, forming an estrogen receptor complex. Unlike anastrozole, tamoxifen's mechanism of action is independent of circulating levels of estrogen. In fact, tamoxifen is effective against ER-positive breast cancer found in premenopausal women [2]. Therefore, even if estrogen levels in our *in vivo* study are more similar to those in premenopausal women than those found in postmenopausal women, we would still expect the drug to elicit an inhibitory effect on 4T1 mouse breast cancer growth. This effect was demonstrated in the present investigation by the observed increase in mouse life span treated with tamoxifen and the combination.

Conversely, no statistical significance was observed in tumor induction time, calculated average tumor volume, or liver and spleen mass at death in any of the treatment groups when compared to untreated mice. One factor that may have contributed to the lack of statistical significance in these areas was probably due to the high number of cells used to induce the tumor. In a subsequent study performed in our laboratory, female BALB/c mice were injected with 1000 and 5000 4T1 cells and tumor development was studied. All mice developed palpable tumors by day 18 of the study. The high metastatic ability of the 4T1 cell line was demonstrated in a study conducted by Pulaski et al [26]. These investigators noted that 4T1 metastasizes to the lungs, livers, and brains of inoculated mice as early as two to three weeks after a 7000 4T1 cell inoculation. Therefore, the initial injection of 10 000 4T1 cells used in our study was most likely too strong in terms of tumor development for differential tumor volume observations for the treatment groups. However, the difference in spleen size noted on day 28 may be an indication of different degrees of 4T1 metastasis occurring across the treatment groups. Tamoxifen treatment may have played a role in prolonging the life of the BALB/c mice by slowing down the metastasis to other areas of the body.

Tumor, spleen, and liver mass at death did not serve as good indicators of drug efficacy because of the differential survival time observed for the mice across the treatment groups. Because mice in the tamoxifen treatment group typically lived longer than mice in the groups that did not receive any of the drugs, these mice had a longer

period of time for primary tumor and spleen enlargement. This could explain the significant difference in tumor mass at death observed between tamoxifen-treated and untreated mice.

Several mice that were near death early in the study were characterized by a swollen abdomen. These mice suffered from ascites, a condition characterized by excess fluid in the abdomen. In a 2002 study, Hasumi et al [27] reported that advanced ovarian cancer often invades the peritoneal cavity resulting in ascites that sometimes contained malignant cells. The development of ascites was shown to inversely affect survival time in humans by Roszkowski et al [28]. Mice observed with abnormally swollen abdomens in this study were determined to be close to natural death and were sacrificed. After dissection, fluid from the abdomen was collected and injected into three BALB/c mice. Tumor formation in all the three mice indicated that the ascites condition occurred as a result of the 4T1 metastasis.

The cell kinetics study conducted *in vitro* revealed that anastrozole, tamoxifen, and the combination of the two drugs elicited a growth inhibitory response in the 4T1 mouse mammary carcinoma cell line. The 50  $\mu\text{g}/\text{mL}$  anastrozole and combination treatments significantly reduced cell viability in each study period when compared to untreated cells, while tamoxifen-treated cells did not differ significantly from untreated cells after 24 hours of drug treatment. These results agree with the quantified cell proliferation results. However, in the qualitative analysis, utilizing both light and scanning electron microscopy, tamoxifen and combination treatments appeared to be more damaging than anastrozole alone in altering 4T1 cell morphology. Furthermore, fewer 4T1 cells were observed per field of view in the tamoxifen and combination treatments when compared to anastrozole or untreated cells. Compared to anastrozole, the cells treated with tamoxifen showed more cell damages such as lysis, irregular cell shapes, blebbing, and prevalence of membrane holes after 24 hours of drug treatment.

A possible explanation for the apparent discrepancy between quantitative and qualitative data might be due to the disruption of cell adhesion leading to detachment of cells from the substratum. In 2002, Palencia et al [29], in their study on thalidomide, suggested that although the agent was not cytotoxic to neoplastic cells, it might have inhibited tumor growth by blocking cell-surface adhesion receptors. A similar mechanism may be in operation with tamoxifen. Tamoxifen might have altered the cell's ability to adhere to the cell surface releasing it into the media without initially killing the cell. In a study conducted by Damiano, integrin cell adhesion molecules were shown to protect myeloma cells from drug-induced apoptosis. They suggested that integrin antagonists capable of isolating tumor cells would be effective in cancer treatment by sensitizing the cell and making the cell more prone to apoptosis [30]. Tamoxifen's reduction of cell adhesion demonstrated *in vitro* may be indicative of interruption of normal integrin reception. The tamoxifen-induced loss of

cell adhesion combined with anastrozole treatment in the cell multiplication study may explain the significant decrease in cell viability observed when the two drugs were administered in combination compared to either agent alone. The adverse effects of the drugs on proteins that are required to maintain cell structures such as intermediate filaments, actin, gelsolin, and laminin might have led to changes in cell shape, cell fragmentations, as well as in formation of cell membrane blebbing and apoptotic bodies [31].

The mitochondrial membrane potential disruption assay was used to detect one of intracellular changes occurring in the activation phase of apoptosis in 4T1 cells [32, 33]. Apoptosis, or programmed cell death, is a mechanism of cell suicide which can be divided into two major phases: the activation and execution phases [31]. A variety of chemotherapeutic drugs initiate apoptosis in tumor cells leading to the regression of a cancerous tumor [32]. In this study, all treatment groups exhibited a higher incidence of apoptosis compared to untreated cells. Estrogen is a known apoptotic inhibitor [34]. It is possible that both anastrozole and tamoxifen directed the cells toward apoptosis by interfering with various estrogen signaling events. In their study, Davis and Majumdar reported that raloxifene, a selective estrogen receptor modulator with anti-breast-cancer potential, showed high incidences of apoptosis through disruption of the mitochondrial membrane [32]. In our study, the highest incidence of apoptosis was seen in the combination treatment of tamoxifen citrate and anastrozole. However, at 72 hours no significant difference was detected between combination- and tamoxifen-treated cells due to an extremely high incidence of cell loss. Since mitochondrial membrane potential disruption was observed in all treatment groups, the results of this assay suggest that the initial mechanism of cell death elicited by anastrozole, tamoxifen, and the combination was triggered via apoptotic pathway.

Surface ultrastructural characteristics detected in the SEM study also suggest that apoptosis may be the mechanism of drug-induced cell death at the initial treatment period. Specific morphological alteration identified in the micrographs of drug-treated cells included apoptotic body formation, cell membrane blebbing, and thinning of lamellipodia; these are characteristics which are indicative of apoptosis [35, 36]. Perry et al used transmission electron microscopy to conclude that apoptosis was induced in human breast cancer cells following exposure to tamoxifen [37].

Efficacies of anastrozole, tamoxifen, and the combination treatments were examined in *in vitro* and *in vivo* settings in the mouse model. While *in vitro* experiments showed a significant growth inhibitory effect in 4T1 mouse breast cancer cells exposed to all treatments, the *in vivo* tumor development study indicated that tamoxifen and the combination treatments extended the survival time for BALB/c female mice. Although the survivability was increased by several days in anastrozole-treated mice, the difference in life span between the anastrozole-treated



and untreated 4T1-inoculated mice was not statistically significant. In vitro assay procedures might suggest that both the drugs and their combination treatments extended cell death via an apoptotic pathway.

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Response of mouse breast cancer cells to anastrozole, tamoxifen, and the combination. Xanthopoulos, J; Romano, A; Majumdar, S. Effect of melatonin on tumor growth and angiogenesis in xenograft model of breast cancer. Jardim-Perassi, BV; Arbab, AS; Ferreira, LC; Borin, TF; Varma, NR; Iskander, A. Blockade of Fas signaling in breast cancer cells suppresses tumor growth and metastasis via disruption of Fas signaling-initiated cancer-related inflammation. Liu, Q; Tan, Q; Zheng, Y; Chen, K; Qian, C; Li, N. Inhibition of spontaneous breast cancer metastasis by anti-Thomsen-Friedenreich antigen monoclonal antibody. This combination testosterone-anastrozole (T + A) implant therapy, delivered locally (intramammary/peritumorally) in the neoadjuvant setting, has several theoretical advantages, including higher doses of both testosterone and the aromatase inhibitor anastrozole being delivered directly to the tumor. Although there was virtually no response to oral tamoxifen therapy between December 2012 and March 2013 (as evidenced by tumor measurements on office US), our patient manifested a rapid clinical response to intramammary T + A therapy by 46 days, continuing through week 13. The combination of testosterone delivered simultaneously with anastrozole allows the