

Effects of Exemestane on Two-Dimensional and Three-Dimensional T47D Breast Cancer Cell Cultures

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Abstract

These studies examined the T47D human ductal breast epithelial tumor cell line in two-dimensional (2D) and three-dimensional (3D) cell cultures with the purpose of comparing the physiological differences between cultures. The cultures were also treated with exemestane, a chemotherapeutic aromatase inhibitor, to compare growth inhibition of the cells under both conditions. The 2D cell cultures were attached to tissue culture plastic surfaces and formed monolayers with typical epithelial characteristics. The 3D cell cultures in 10% collagen gels portrayed circular characteristics and formed masses of cells within the collagen suggesting tight colony formation. The growth rate of the cells in 2D cultures was greater than its complement in 3D cultures. The viability of the 2D cell cultures were not inhibited by exemestane, while the 3D cells were significantly inhibited in the presence of 250 nM exemestane. Our research therefore shows a novel difference in the growth rate and inhibition of exemestane in two-dimensional and three-dimensional T47D cell cultures.

Introduction

Two-dimensional cell culturing on flat plastic surfaces is the current standard technique for cell culturing, but presents many limitations when executing cellular research. Three-dimensional cell culturing in a medium that allows cells to produce floating cultures provides a platform which better emulates *in vivo* environments. This system can be provided by culturing the cells in a collagen matrix, which mimics the extracellular matrix and is therefore closer to natural cellular conditions. These conditions give researchers the ability to study patterns of gene expression and other biological activities that better mimic the natural environment in living organisms.¹

The T47D human ductal breast epithelial tumor cell line expresses nuclear estrogen receptors, which are required by the cell to activate certain genes essential for cell growth and replication.² Estrogens are a class of sex hormones consisting of estradiol, estrinol, and estrone. These hormones have the ability to cross cell membranes, giving them the capability to diffuse directly into the nucleus. Once estrogens enter the nucleus, they bind to the substrate-binding domain of the estrogen receptor causing the formation of a receptor dimer. The DNA-binding domain of the receptor then binds to specific sites on DNA and up- or down-regulates gene expression depending on the role of the transcription factor binding site.³

Aromatase is a cytochrome P450 enzyme responsible for the aromatization of estrogens from

androgens. It is localized in the endoplasmic reticulum and consists of a polypeptide chain of 503 amino-acid residues and a heme group. In a three-step process requiring O₂, NADPH, and cytochrome P450 reductase, aromatase converts androstenedione to estrone and testosterone to estradiol by the removal of the 19-methyl group.⁴

Aromatase inhibitors (AIs) block the production of estrogens through competitive binding of the aromatase active site. AI drugs were approved by the FDA in the early 2000s and are currently utilized in the treatment of hormone receptor positive breast cancer in post-menopausal women. There are currently two types of AI drugs approved to treat breast cancer: reversible non-steroidal inhibitors and irreversible steroidal inhibitors.

The reversible non-steroidal inhibitors include anastrozole and letrozole (triazole derivatives). These non-steroidal inhibitors interact with the heme group of aromatase and act as competitive inhibitors; but due to the reversible binding of non-steroidal inhibitors, continual presence of the triazole derivatives is required for prolonged inhibition of aromatase.⁵

Exemestane (6-methylenandrosta-1,4-diene-3,17-dione) is a third generation irreversible steroidal aromatase inhibitor. Exemestane binds to the substrate-binding site of aromatase (residues 309-Asp and 359-Met), while hemoglobin (residue 437-Cys) acts as a co-factor catalyzing the irreversible reaction.

This reaction causes aromatase to denature and degrade due to exemestane analogous structure to the natural substrate androstenedione.⁶ Exemestane is produced under the trade name AROMASIN® by Pfizer Inc.

The purpose of this research was to compare the structure, the growth, and the inhibition of T47D cells in two-dimensional and three-dimensional cell cultures

Materials and Methods

T47D cell culturing: Two-dimensional cultures were grown as monolayers on flat plastic surfaces. Three-dimensional cultures were grown as floating cultures in 24-well plates by utilizing 1 ml of suspended cells in growth medium and 0.5 ml of 10% collagen gel per well. The 10% collagen gel was produced by mixing type A gelatin (from porcine skin) in deionized water. The mixture was then autoclaved at 121 °C at 15 psi for 15 minutes to ensure sterility. The collagen was then aliquoted into 24-well plates, wrapped with Parafilm, and stored at 4°C. Both cultures utilized Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U penicillin, and 0.1 mg/ml streptomycin growth medium.

Microscopy: An inverted microscope was utilized to view and photograph cells at 40x, 100x, and 200x. Photos of the cells were obtained with an OptixCam Summit Series: OCS-1.3 MP Microscope Camera utilizing OptixCam OCview version 7.1 software on a laptop computer. ImageJ program was utilized to create three-dimensional surface plots from stacks of photos at slightly different focal planes.

Growth curve: T47D cells were cultured in a 24-well plate, which contained collagen gel and non-collagen wells. After 48, 96, and 144 hours of incubation, cells were removed from the wells and a Coulter Z1 particle counter was utilized to quantitate the cells present.

Exemestane Dose Response Curve: T47D cells were cultured in either 48- or 24-well plates. Once the cells had grown to 50% confluence, the media was replaced with Opti-MEM (1X) + GlutaMAX-1 to starve the cells overnight. The cells were treated with doses of exemestane ranging from 1 to 20,000 nM in Opti-MEM with 1% DMSO. After 24 and 48 hours of treatment, the MTT assay was utilized to determine cell viability. The effective dose that produces an effect in 50% of the population (ED₅₀) was determined with Microsoft Excel.

MTT assay: The MTT assay is a colorimetric assay used to measure cell proliferation. MTT (3-(4,5-

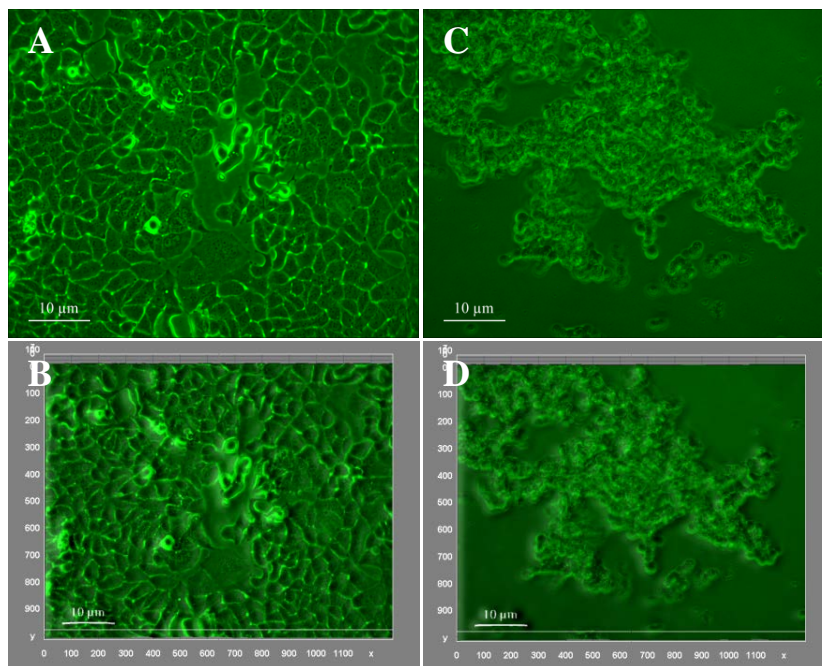


Figure 1. Photographs of T47D cells at 100x, scale represents 10µm. A, two-dimensional T47D cell culture after 264 hours form a monolayer on plastic and display epithelial characteristics. B, ImageJ three-dimensional surface plot of two-dimensional culture. C, three-dimensional T47D cell culture after 24 hours form groupings embedded in the collagen gel and display circular characteristics. D, ImageJ three-dimensional surface plot of three-dimensional culture.

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt which is reduced into an insoluble purple colored formazan precipitate by metabolically active cells. The absorbance of the purple formazan precipitate was quantified at 570 nm.

Results

In two-dimensional cultures, T47D cells formed monolayers and displayed epithelial characteristics. These characteristics included branched cytoplasm and very densely packed cells (Fig. 1A). The cells formed monolayer sheets, in which the cells are attached to each other via tight junctions and desmosomes (Fig. 1B).

In three-dimensional cultures, T47D cells formed small groups within the collagen gels and displayed circular characteristics (Fig. 1C). These groups of cells are interspersed throughout the gel evident by different focal planes (Fig. 1D). The T47D cells in collagen gels were smaller than their counterparts in two-dimensional cultures and tended to congregate toward the edges of the collagen gels. After inoculation the cells began to migrate through the gels and eventually attached to the bottom of the well. The attachment of cells, evident by the presence of epithelial-like cells, took anywhere from 24 to 144 hours after inoculation.

The doubling time of T47D cells cultured on tissue culture plastic between 48-96 hours was 56.61 hours and 96-144 hours was 74.71 hours. The slowed growth of the two-dimensional cultures after 96 hours of incubation may be due to contact inhibition (Fig. 2).

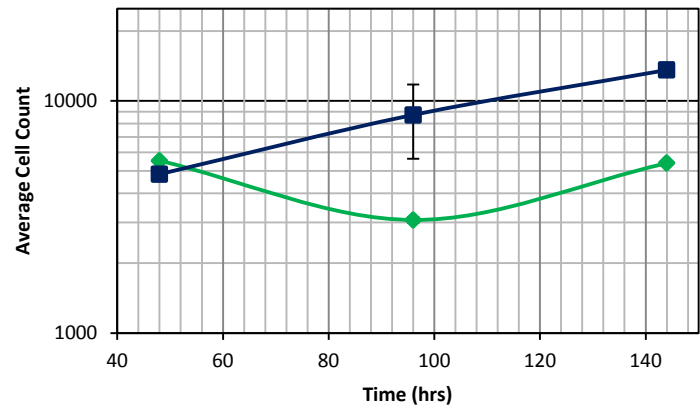


Figure 2. Growth curve comparison of T47D cells cultured in 10% collagen gels (green) and on plastic (blue). The cells cultured two-dimensionally on plastic replicated at a steadier rate than cells cultured in collagen.

The doubling time of T47D cells cultured in collagen gels between 96-144 hours was 59.12 hours. The initial high count of T47D cells cultured in collagen gels could be attributed to dead cells which remained in the collagen gels after inoculation (Fig. 2).

The ED₅₀ of T47D cells cultured in collagen gels was 80 nM after 24 hours and 65 nM after 48 hours (Fig. 3). The T47D cells cultured two-dimensionally were not significantly inhibited by exemestane. The most effective dose of exemestane for the three-dimensionally cultured T47D cells was 250 nM (Fig. 3).

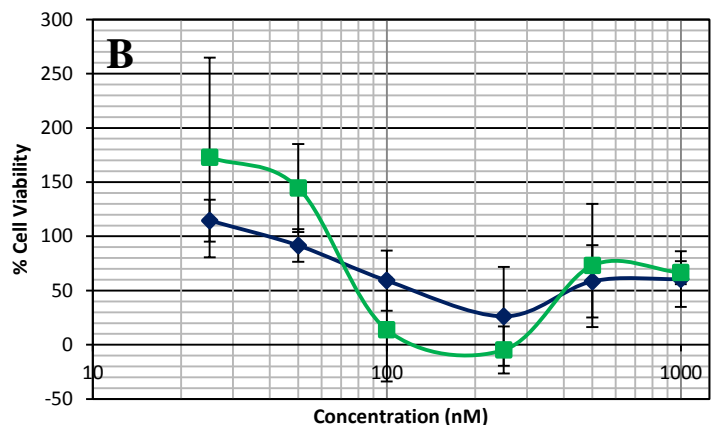
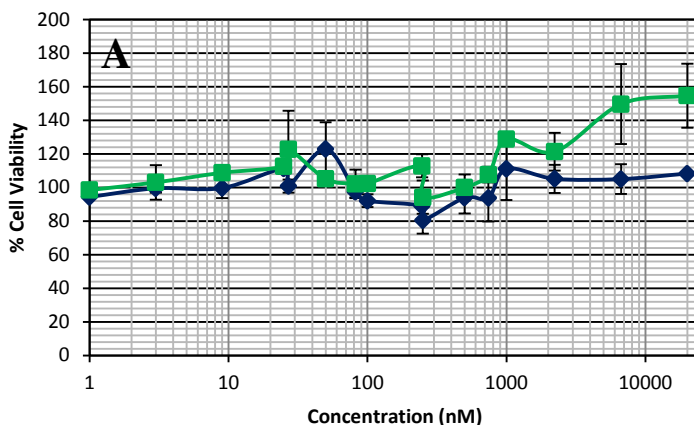


Figure 3. Dose curves of exemestane treated T47D cells after 24 hours (blue) and 48 hours (green). The MTT assay was utilized to measure percent cell viability as compared to control T47D cells. Cells were cultured in the absence of phenol red. A, Plot of two-dimensional cell cultures after exemestane treatment. The cells were not significantly inhibited by 1 to 20,000 nM exemestane. B, Plot of three-dimensional cell cultures after exemestane treatment. The cells were significantly inhibited by 250 nM exemestane.

Discussion

The T47D cell line and many other cells lines have traditionally been cultured as monolayers due to ease of available methods. To better mimic *in vivo* conditions cells can be grown as three-dimensional cultures. The T47D cells cultured in 10% collagen gels were circular due to the lack of attachment to the collagen matrix. The three-dimensional cells appeared to be smaller in size than the cells in monolayers. This may be explained by the ability of the two-dimensional cells to spread out on flat surfaces, which gives it the appearance of being larger.

Here, we demonstrate that the T47D cell line can be grown as three-dimensional cultures in 10% collagen gel; however, the cultures have an initial lag period compared to two-dimensional T47D cultures. This initial lag period may be attributed to dead cells in the collagen gels. Any dead cells in the two-dimensional cultures were removed during the trypsinizing of cells. After 96 hours the three-dimensional cultures proliferate at the same rate as two-dimensional cultures do prior to contact inhibition.

To ensure the absence of interference from estrogen and phenol red during testing, DMEM/F12 growth media was changed before the treatment of the cells. Phenol red is a weak estrogen mimic, and in cell cultures that express the estrogen receptor it can enhance growth.⁷

The T47D cell line was determined to produce aromatase by the presence of aromatase mRNA by Northern hybridization.⁸ Three-dimensional T47D cultures were inhibited by exemestane; however, two-dimensional T47D cultures were not significantly inhibited. This suggests three-dimensional T47D cultures are producing their own estrogens and two-dimensional T47D cultures do not produce or require estrogens as a growth factor.

Researchers have shown the T47D cell line has the ability to become estrogen receptor negative when grown in an estrogen-free environment.² This ability could explain the inhibition difference by exemestane between the two-dimensional and three-dimensional T47D cultures. T47D cells cultured as monolayers may express a cell to cell interaction, which overcomes the requirement of estrogen or estrogen receptor to proliferate. Three-dimensional cultures do not form as closely packed cells as two-dimensional monolayers; consequently, the three-dimensional cultures would not form this cell to cell interaction.

Conclusion

One limitation during this research was the acquisition of cells from the collagen gels. The centrifugation and removal of the collagen from the cultures could be a significant source of error. Future research will compare the estrogen production of two-dimensional and three-dimensional T47D cultures. If two-dimensional T47D cultures express estrogen, then a novel pathway which overcomes the requirement of estrogen in two-dimensional cultures will be studied.

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References

1. Wozniak M., Keely P. "Use of three-dimensional collagen gels to study mechanotransduction in T47D breast epithelial cells." *Biol. Proced. Online* 2005; 7(1): 144-161.
2. Murphy CS, Meisner LF, Wu SQ, Jordan VC. "Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture." *Eur J Cancer Clin Oncol.* 1989; 25(12): 1777-1788.
3. Katzenellenbogen B, Katzenellenbogen J. "Estrogen receptor transcription and transactivation: Estrogen receptor alpha and estrogen receptor beta - regulation by selective estrogen receptor modulators and importance in breast cancer." *Breast Cancer Res.* 2000; 2(5): 335-344.
4. Ghosh D, Griswold J, Erman M, Pangborn W. "Structural basis for androgen specificity and oestrogen synthesis in human aromatase." *Nature* 2009; 457(7226): 219-223.
5. Wang X, Chen S. "Aromatase Destabilizer: Novel Action of Exemestane, a Food and Drug Administration -Approved Aromatase Inhibitor." *Cancer Res* 2006; 66(21): 10281-10286.

6. Geisler J, King N, Anker G, Ornati G, Di Salle E, Lønning PE, Dowsett M. "In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients." *Clin Cancer Res.* 1998; 4(9): 2089-93.
7. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. "Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture." *Proc Natl Acad Sci USA.* 1986; 83(8): 2496-2500.
8. Sadekova SI, Tan L, Chow TY. "Identification of the aromatase in the breast carcinoma cell lines T47D and MCF-7." *Anticancer Research* 1994; 14(2A): 507-11.

Biography

Bryan Neumann is currently enrolled as a senior at the University of New Haven majoring in Forensic Science, Biochemistry, and Biotechnology. He is planning to attend graduate school to obtain a Ph.D. in Cellular and Molecular Biology. Afterwards, he would like to conduct industrial or academia cancer research.

His hometown is Sparta, NJ and he is a brother of the Sigma Chi Fraternity. In his spare time he likes to listen to music, watch soccer, and take photographs.



