

The Effects of Curcumin on ER α , p53, and p21 in the MCF-7 Breast Cancer Cell Line

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Abstract

Curcumin, a golden-yellow flavonoid derived from turmeric, has been used in traditional Chinese and Indian medicine for centuries due to its antioxidant and anti-inflammatory properties. Recent interest has grown in its potential therapeutic applications in cancer research, particularly breast cancer, which is the most commonly diagnosed cancer and the second leading cause of cancer death in women. This study investigates the effects of curcumin on the MCF-7 breast cancer cell line. MCF-7 cells were cultured in charcoal-stripped serum and treated with curcumin, alone and in combination with hormones and antihormones. After performing protein extraction and Bio-Rad Bradford protein assay, SDS-PAGE gel electrophoresis was performed followed by western blot analysis. Cellular viability was assessed using Propidium Iodide (PI) staining and confocal microscopy. Statistical analyses included Kruskal-Wallis testing and post-hoc Mann-Whitney U-tests, conducted using SPSS software. Western blot analysis revealed significant changes in ER α , p53, and p21 protein levels after 24-hour treatment with 5–100 μ M curcumin. Specifically, curcumin induced an 80% reduction in ER α expression at 40 μ M, and increased p53 and p21 levels by 40% and 80%, respectively. A 36% decrease in cellular viability was noted after six days of 40 μ M curcumin treatment. Co-treatment with curcumin and E2 resulted in a 79% downregulation of ER α and increased p53 and p21 expression by 15% and 94%, respectively, compared to control. E2's proliferative effect was mitigated by curcumin, with no significant changes in ER α cytolocalization observed. Curcumin shows potent anti-cancer properties in the MCF-7 breast cancer cell line, potentially contributing to understanding its molecular effects on breast cancer progression. This study supports further investigation into curcumin as a therapeutic agent in breast cancer treatment.

Keywords: Tumor suppressor; MCF-7; Antitumor agent; Natural anticancer; Cancer treatment.

Introduction

Breast cancer is the most commonly diagnosed cancer in women, impacting millions annually. With diverse risk factors and intricate molecular pathways involved in its progression, understanding the complexity of breast cancer is crucial for advancing prevention, early detection, and treatment strategies. In the year 2020, an estimated 2.3 million novel instances of

breast cancer were diagnosed globally [1]. The mortality landscape of breast cancer continues to be a substantial global health challenge; in 2020, breast cancer led to approximately 42,170 deaths in the United States alone [2]. Estrogen, a primary sex hormone in females, plays a crucial role in the regulation of various physiological processes, including the development and maintenance of secondary sexual characteristics. In the

context of breast tissue, estrogen exerts proliferative effects on epithelial cells, contributing to the intricate balance of cellular growth and differentiation. A comprehensive understanding of estrogen's function in breast tissue is essential for unraveling its implications in both normal physiological states and pathological conditions such as breast cancer [3,4].

Estrogen has demonstrated in previous laboratory studies, as well as studies from our laboratory, proliferative effects on the hormone-dependent MCF-7 and T-47D breast cancer cell lines [3,5-8]. The primary receptor for E_2 is estrogen receptor alpha, or ER α . In healthy breast epithelium, this receptor is present only 10% of the time, but when diseased breast epithelium is studied, it is shown to be present in approximately 50-80% of breast tumors. The presence of ER α significantly increases breast tumor mitotic activity in patients with mutated tumor-suppressor genes [9-10]. Furthermore, abnormal ER α expression has been shown to play a role in the development and progression of hormone-dependent breast cancer [10,11].

The MCF-7 breast cancer cell line used in this study was isolated from an infiltrating ductal carcinoma of the breast; the cell line is estrogen receptor positive and has the wild-type p53 gene [12]. The tumor suppressor protein p53 halts the cell cycle when DNA damage is found, induces apoptosis if damage is beyond repair, and ultimately protects cells from cancerous mutations. In approximately 50% of cancers, the TP53 gene is mutated, leading to unregulated cancer cell proliferation and progression [13]. The p21 protein is a cyclin-dependent kinase (cdk) inhibitor, which is induced by p53 in instances of DNA damage or oxidative stress [14,15]. As research advances, it seems that p21 may have a dual role as an oncogene and tumor suppressor; nevertheless, an upregulation of p21 has been shown to be synergistic with cancer treatment according to the literature [14,16]. Due to the intricate linking of these genes, proteins, and cancer progression, they are imperative to study in accordance with ER α to understand the progression of breast cancer.

Curcumin is a golden-yellow flavonoid compound derived from the turmeric (*Curcuma longa*) plant root which has been used in Chinese and Indian medicine for centuries. Its appeal to cancer research and therapeutics lies in its demonstrated antioxidant, anti-inflammatory, and anti-cancer properties [17-19]. Studies have shown curcumin to exhibit anticancer effects in Cancer Stem-Like Cells (CSCs), and also to restore expression of E-cadherin, a tumor suppressor, in breast CSCs [20,21]. Many other studies have also shown that curcumin suppresses breast cancer cells in vitro, leading to a concentration-dependent decrease in cancer cell growth, through a proposed mechanism of p53-induced apoptosis [22,23]. Our laboratory previously studied the effects of curcumin on the T-47D breast cancer cell line and found that curcumin significantly downregulated both p53 and ER α protein levels with a concomitant decrease in cell viability [24]. In this study, we investigate the effects of curcumin on ER α , p53, and p21 expression in the presence of hormones, and antihormones on the MCF-7 breast cancer cell line. This study will further contribute to the knowledge of curcumin's anticancer properties and its actions on a molecular level in the proliferation and progression of breast cancer.

Materials and methods

Cell culture and treatment : The hormone-dependent MCF-7 breast cancer cell line (ATCC[®] HTB-133 TM; ATCC, Manassas, VA, USA) was regularly cultured at 37°C with 5% CO₂, which

mimics the ideal condition of the human body. The cells were cultured in Eagle's minimum essential medium (Corning; Corning, NY, USA), which contained 1.5 g/L sodium bicarbonate, nonessential amino acids, l-glutamine, and sodium pyruvate. The cells were then plated in six-well plates (240,000 cells per well), and the medium was transitioned to a 5% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS; Santa Cruz Biotechnology; Dallas, TX, USA) (5% stripped serum) to avoid cellular exposure to endogenous steroids and growth factors within the FBS. This stripped serum allowed the cells to be at their basal metabolic rate at the time of treatment, confirming that any effects seen on the cells and the cells' proteins are caused exclusively by the treatments they receive and not by any other components of the culturing media. The cells were cultured in this charcoal-stripped serum for 6 days. On the sixth day, the cells were treated with 2 μ L of ligands for 24 hours. Control groups were treated with dimethyl sulfoxide (DMSO), the solvent used for curcumin treatments. Varying concentrations (5-100 μ M) of curcumin were used in the concentration-dependent studies, while estrogens and antiestrogens were combined with 40 μ M curcumin for the hormone studies. For estrogen treatments, 10 nM E_2 was used. Additional treatments include a pure estrogen antagonist, 1 μ M ICI (ICI 182, 780), and a selective estrogen receptor modulator, 1 μ M TAM (Tamoxifen), was utilized.

Protein extraction and quantification

The cellular proteins were extracted with radioimmunoprecipitation assay buffer obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The cells were then centrifuged at a speed of 15,000 \times g for 15 minutes at 4°C. After centrifugation, the protein supernatant was separated and used to prepare a protein assay based on the Bradford method (Bio-Rad; Hercules, CA, USA), which allowed for the quantification and normalization of the protein in each extracted sample by use of a spectrophotometer.

SDS-PAGE and western blot analyses

The extracted proteins were subjected to SDS-PAGE, prepared with SDS detergent and β -mercaptoethanol and followed by heating the supernatant for 3 minutes at 85°C. Equal amounts of protein were then loaded into a 7.5%–12.5% polyacrylamide gel and transferred to an Immobilon polyvinylidene fluoride membrane (Millipore; Bedford, MA, USA) by the process of electroblotting. To begin probing, 5% nonfat dry milk was used to block nonspecific proteins. The membranes were then probed with the corresponding primary and secondary antibodies for each protein of interest. For ER α , anti-ER anti-mouse monoclonal antibody (1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used. To detect the primary antibody, a secondary goat anti-mouse IgG2a antibody (1:2000) was used. Anti-p53 anti-mouse monoclonal (1:2000) and anti-p21 BSA 5xTBS monoclonal (1:2000) primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and detected by secondary goat anti-rabbit antibodies (1:2000). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The specific band for each protein of interest was then visualized by the enhanced chemiluminescence method according to the instructions from Advansta (San Jose, CA, USA) and the Bio-Rad Chemi-Doc XRS + imaging system. The Western blots were subjected to quantification of the protein band density using the Image Studio Lite program, version 3.1 (LI-COR Biosciences; Lincoln, NE, USA).

Cell viability assay

Our cell viability assays demonstrate the number of live cells in a total population after treatments with ligands at varying concentrations for 6 days, with treatments administered every 2 days. The cell viability studies were cultured in 12-well plates (30,000 cells per well). Throughout the experiment, the medium was replenished every 48 hours. The plates were fed with 10% whole serum for the first 2 days to ensure confluency. The cells were then incubated in the presence of stripped serum for 6 days and quantified on the sixth day using the Cellometer Vision CBA Image Cytometry System software version 216 (Nexcelom Bioscience LLC.; Lawrence, MA, USA). This was accomplished via PI staining, which fluorescently tags dead cells. The number of dead cells versus total cells was quantified in order to calculate cell viability.

Immunofluorescence and confocal microscopy

Cells were plated onto coverslips in 12-well plates (30,000 cells per well) with the appropriate medium for the cell line. Subsequently, the cells were incubated with stripped serum for 6 days, and immunolabeling was performed for ER α in MCF-7 cells. The distribution of three-dimensional fluorescent structures was analyzed using a Nikon Digital Eclipse C1-Plus confocal microscope (Nikon Instruments; Melville, NY, USA) and differential interference contrast (DIC) images were taken in parallel. NIS Elements AR software (Nikon Instruments) was used for image analysis.

Statistical analyses

The results are expressed as mean \pm SEM. Statistical significance was determined by the Kruskal–Wallis test, followed by post hoc analysis using the Mann–Whitney U test. Differences are considered significant at $P < 0.05$. In all figures: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistical analyses were carried out using SPSS for Windows version 11.5 (SPSS Inc.; Chicago, IL, USA).

Results

Concentration-dependent effects of curcumin on ER α , p53, and p21 protein expression

Figures 1A, 1B and 1C represent the results of the concentration-dependent effects of curcumin on ER α , p53, and p21 expression respectively. Western blot analysis revealed a concentration-dependent decrease in ER α protein expression with treatments of 5–100 μ M curcumin. An 80% decrease in ER α expression was observed with treatment of 40 μ M curcumin when compared to control (Figure 1A). A concentration-dependent increase in expression of both p53 and p21 was observed after treatment of 5–100 μ M curcumin. A 130% increase in p53 protein levels was seen after treatment with 100 μ M curcumin and compared to control (Figure 1B). Expression of p21 was increased by 80% when compared to control after treatment with 80 μ M curcumin (Figure 1C).

Effects of curcumin in combination with estrogens and anti-estrogens on ER α , p53, and p21 protein expression

Figures 2A, 2B and 2C represent the effects of hormonal studies with curcumin on ER α , p53 and p21 expression, respectively. Treatments of E $_2$ (10 nM) show an expected decrease in ER α expression in the MCF-7 cell line (Figure 2A). Similar effects are seen with ICI, as well as the combination of E $_2$ and ICI. TAM

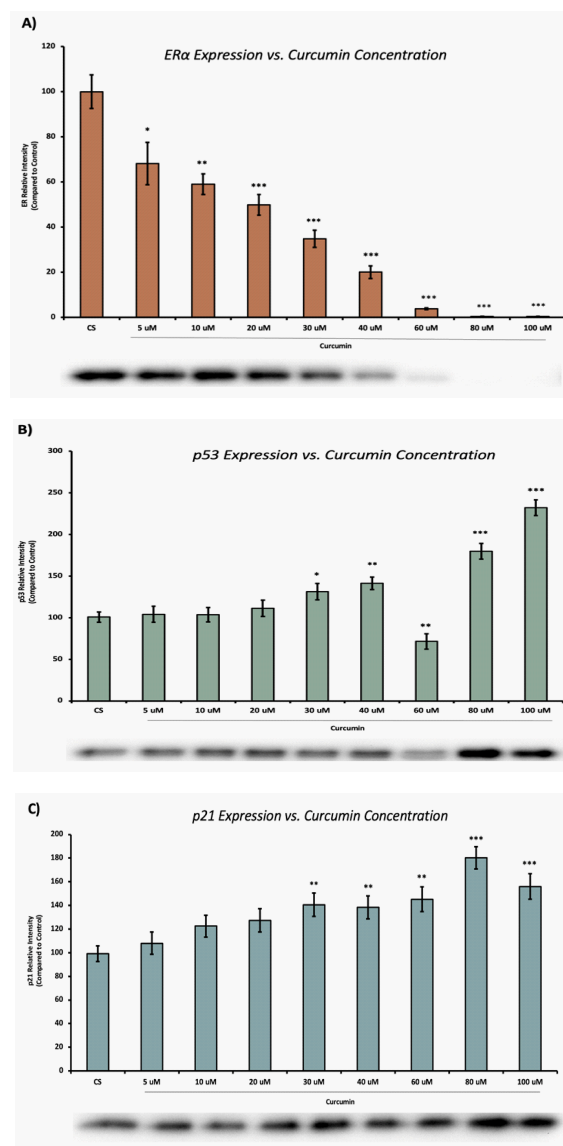


Figure 1: Concentration-dependent effects of curcumin in the MCF-7 cell line on (A) ER α levels (B) p53 levels, and (C) p21 levels. The cells were treated for 24 h with varying concentrations of curcumin (5 μ M–100 μ M) and subjected to SDS-PAGE and Western blot analyses, as described in Section 2.3. Protein band densities were quantified using Image Studio Lite. Significance levels were determined based on the following thresholds: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistical analyses were performed using SPSS (Windows Version 11.5, SPSS Inc.; Chicago, IL, USA).

showed an expected increase in ER α expression, which normalized compared to control with curcumin co-treatment. A synergistic effect was observed on the downregulation of ER α expression when curcumin (40 μ M) was combined with E $_2$ or ICI. An 80% decrease in ER α expression was observed following treatment of curcumin and E $_2$. Combined treatments of curcumin and E $_2$ showed no significant effect on p53 when compared to control (Figure 2B). However, when curcumin was combined with ICI, TAM, and Bisphenol S (BPS), significant reduction in p53 expression was observed when compared to control (Figure 2B). A maximum 60% reduction in p53 expression was noted when curcumin was combined with BPS. Treatment of curcumin and E $_2$ resulted in a 94% increase in p21 when compared to the control (Figure 2C). Similarly, when curcumin was combined with ICI a 94% increase in p21 was also demonstrated when compared to the control.

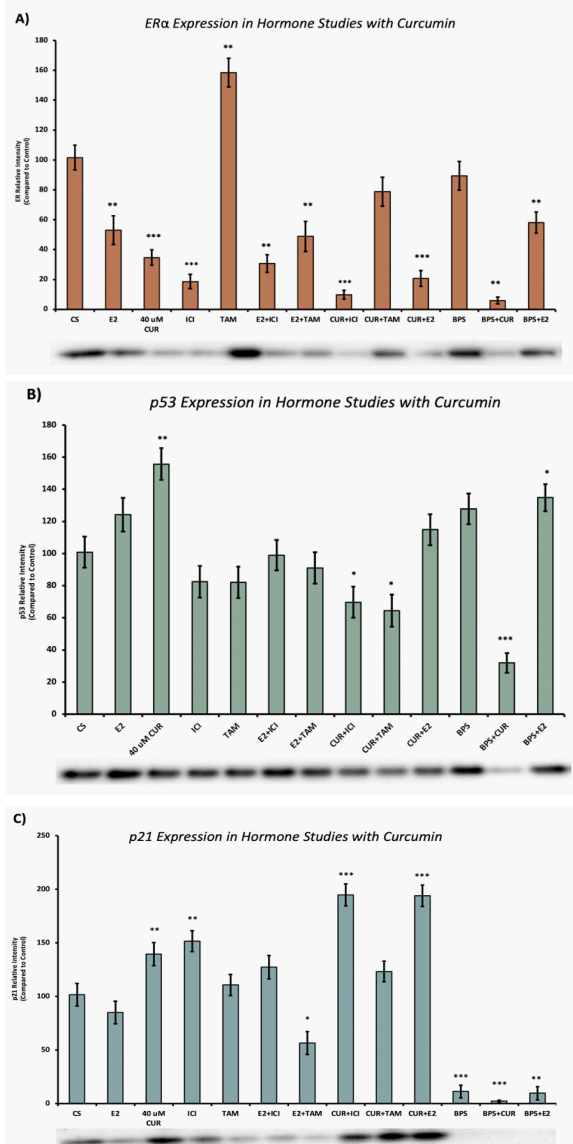


Figure 2: Hormonal and antihormonal effects of curcumin (CUR) in the MCF-7 cell line on (A) ER α levels, (B) p53 levels, and (C) p21 levels. The cells were treated for 24 h with varying combinations of curcumin (40 μ M), hormones, and antihormones and subjected to SDS-PAGE and Western blot analyses, as described in Section 2.3. Protein band densities were quantified using Image Studio Lite. Significance levels were determined based on the following thresholds: * p <0.05, ** p <0.01, and *** p <0.001. Statistical analyses were performed using SPSS (Windows Version 11.5, SPSS Inc.; Chicago, IL, USA).

Effects of curcumin in combination with estrogens and anti-estrogens on cell viability

Treatment with E₂ displayed a proliferative effect on MCF-7 cell viability, which is to be expected. This effect of E₂ was reversed when cells were treated with a combination of E₂ and curcumin, which showed a 62% decrease in cellular proliferation when compared to control (Figure 3). Similarly, the proliferative effect of BPS on MCF-7 cells was reversed with co-treatment of BPS and curcumin, which demonstrated a 69% decrease when compared to the control.

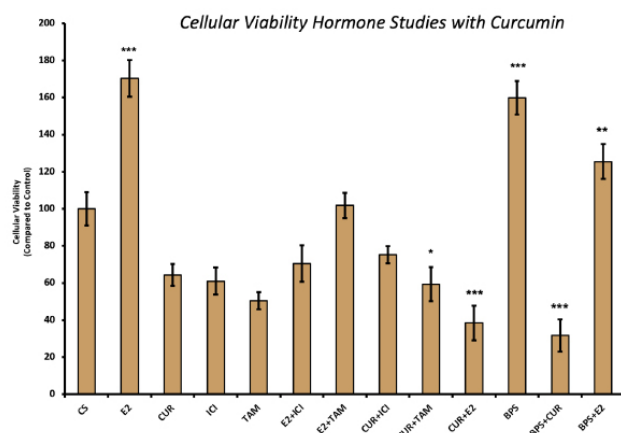


Figure 3: Hormonal and antihormonal effects of curcumin (CUR) on cell viability in the MCF-7 cell line. The cells were treated for 6 days with varying combinations of curcumin (40 μ M), hormones, and antihormones. Cell viability was determined via PI staining and image cytometry using the Cellometer Vision CBA Image Cytometry System, following the procedures outlined in Section 2. For analysis purposes, the collected samples were compared to the control group. Significance levels were determined based on the following thresholds: * p <0.05, ** p <0.01, and *** p <0.001. Statistical analyses were performed using SPSS (Windows Version 11.5, SPSS Inc.; Chicago, IL, USA).

Effects of curcumin on the cellular localization of ER α

Confocal images show that ER α is localized in the control (DMSO) intranuclearly, as expected (Figure 4). Following treatment of cells with 10 nM E₂, 1 μ M ICI, 40 μ M curcumin, TAM, BPS and combinations of these, there is no observed change in cellular localization of ER α .

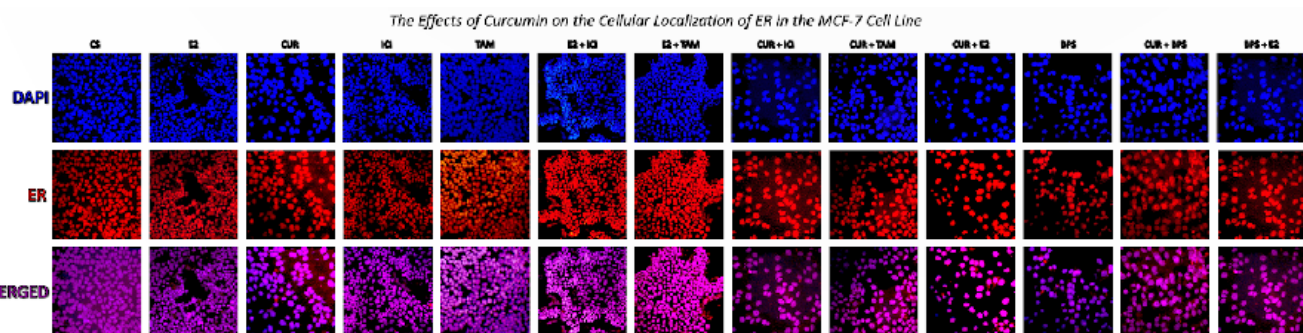


Figure 4: Effects of curcumin (CUR) on the cellular localization of ER α in the MCF-7 cell line. Images were acquired as described in Section 2. The presented results depict the effects on cells exposed to Cy3 and DAPI immunofluorescence stains, corresponding to the row of red-hued and blue-hued images respectively. The row of purple-hued images illustrates the merged overlay of these two staining techniques.

Discussion

Breast cancer remains a pervasive global health challenge, with millions of cases diagnosed annually and significant mortality rates, emphasizing the urgency for enhanced prevention, early detection, and treatment strategies [1,2]. The pivotal role of estrogen, particularly through its proliferative effects on breast tissue, underscores the intricate balance in cellular growth and differentiation, with abnormal ER α expression implicated in hormone-dependent breast cancer development and progression [3,4]. Notably, the interplay of key regulators such as ER α , p53, and p21 in breast cancer pathogenesis is underscored, with mutations in these genes contributing to unregulated cell proliferation. Curcumin, derived from the turmeric plant, has garnered attention for its profound antioxidant, anti-inflammatory, and anti-cancer properties. Extensive research suggests curcumin's potential to inhibit cancer cell growth, particularly in breast cancer, and modulate key molecular pathways involved in proliferation [17-21]. Previously, our laboratory studied the effects of curcumin on the T-47D breast cancer cell line and found curcumin to significantly downregulate both p53 and ER α protein levels with a concomitant decrease in cellular viability [24]. Our current studies expand on this investigation and contribute to the understanding of curcumin's mechanism of action on the hormone-dependent breast cancer cell line, MCF-7. Overall, western blot analysis demonstrates a concentration-dependent decrease in ER α expression with increasing curcumin concentrations, depicting an 80% reduction in expression when the optimum concentration of 40 μ m curcumin is compared to the control. This effect is maintained upon co-treatment of E₂ and curcumin, which again demonstrates an 80% decrease in ER α expression when compared to the control. This is consistent with our findings in the cellular viability experiment, which depicts a corresponding 62% decrease in cellular viability following the same treatment combination. This may indicate the relationship between ER α expression and breast cancer proliferation. In addition, ER α expression is significantly decreased when curcumin is combined with the antiestrogen ICI, even more so than when either compound is used in isolation. In addition, there is no observed change in cellular localization of ER α following hormone treatment. These effects of curcumin on ER α expression are consistent with the results seen in our previous studies within the T-47D cell line [24].

The protective, anti-cancer effects of tumor suppressor gene TP53 have been well documented, along with the mechanism of its downstream effector protein p21 [13-15]. In our concentration studies, curcumin treatment demonstrates a concentration-dependent increase in both p53 and p21 levels when compared to the control, with a maximum 132% and 80% increase in each protein respectively. This increase in p21 expression was sensitive to the co-treatment of curcumin and E₂, demonstrating a 94% increase when compared to the control. Additionally, when treated with combinations of curcumin and antiestrogens ICI and TAM, this downstream effect on p21 still prevails, with demonstrated 94% and 23% increase respectively. This portrays the continuous protective effects of curcumin even in the anti-estrogen setting. These results are corroborated by the corresponding decrease in cellular viability when curcumin is combined with antiestrogens ICI and TAM; cellular viability decreased by 25% and 41% after these respective treatments when compared to the control. Our results regarding the p53 studies in the MCF-7 cell line are in contrast to our findings previously in the T-47D cell line, which displayed a concentration-

dependent decrease in p53 levels following curcumin treatment [24]. This contradiction is likely because the T-47D cell line harbors a TP53 gene mutation while the MCF-7 cell line's TP53 gene is wild-type.

Moreover, estrogen treatment alone has been well documented to increase the proliferation of breast cancer cells, which is depicted in our cellular viability experiment by a 70% increase in viability following treatment of E₂ when compared to the control [5-8]. However, when curcumin and E₂ are combined as co-treatment, curcumin's antiproliferative effects are able to suppress the proliferative effects of E₂, which is depicted by the 62% decrease in cellular viability of this combination. This effect is even more dramatic than the decrease in cellular viability noted when cells are treated with TAM or ICI in isolation.

On a final note, our findings contribute to the current discussion of breast cancer treatment and prevention on the molecular level; They offer an improved understanding of the effects of curcumin not only on ER α , but also on p53 and p21 expression, and their corresponding effects on cellular viability. Our studies delineate the clear anti-estrogenic, and anti-cancer nature of curcumin, and corroborate its protective effects on an additional breast cancer cell line, MCF-7. The combination of decreased ER α expression, increased p53 and p21 expression, and decreased cellular viability following curcumin treatment is promising in terms of cancer treatment and/or prevention. Moreover, the fact that these effects are maintained in the setting of estrogenic and anti-estrogenic compounds is even more encouraging. Further studies are indicated to expand upon this research in the clinical setting.

Declarations

Conflicts of interest: The authors declare no conflicts of interest.

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publication: Informed consent to publication was obtained from relevant participants.

Availability of Data and Materials: The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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