

The effects of turmeric (curcumin) on tumor suppressor protein (p53) and estrogen receptor (ER α) in breast cancer cells

Kelly Hallman
Katie Aleck
Brigitte Dwyer
Victoria Lloyd
Meghan Quigley
Nada Sitto
Amy E Siebert
Sumi Dinda

School of Health Sciences, Prevention
Research Center, Oakland University,
Rochester, MI, USA

Abstract: Curcumin (CUR) is a compound that has antibacterial, antiviral, anti-inflammatory, and anticancer properties. In this study, we have analyzed the effects of CUR on the expression of ER α and p53 in the presence of hormones and anti-hormones in breast cancer cells. Cells were cultured in a medium containing charcoal-stripped fetal bovine serum to deplete any endogenous steroids and treated with CUR at varying concentrations or in combination with hormones and anti-hormones. Protein analysis revealed a relative decrease in the levels of p53 and ER α upon treatment with 5–60 μ M CUR. In cell proliferation studies, CUR alone caused a 10-fold decrease compared with the treatment with estrogen, which suggests its antiproliferative effects. Delineating the role of CUR in the regulation of p53, ER α , and their mechanisms of action may be important in understanding the influence of CUR on tumor suppressors and hormone receptors in breast cancer.

Keywords: breast cancer, tumor suppressors, p53, estrogen, anti-estrogens, female sex steroids, turmeric

Introduction

In the USA alone, breast cancer is the second leading cause of cancer death in women.¹ Invasive breast cancer (IBC) is known to develop after the progression of multiple stages, one of the later stages being referred to as ductal carcinoma in situ (DCIS). DCIS is an intraductal neoplastic proliferation of epithelial cells that do not invade through the basement membrane layer to the breast stroma.^{1–3} In several cases, it is known to be preliminary to IBC, and it has been suggested that the proliferation of DCIS is dependent on the presence of estrogen. Once the DCIS becomes cancerous, it is known as ductal carcinoma, which is a form of hormone-dependent breast cancer.^{1–3} The T-47D cell line used in our experiments was isolated from infiltrating ductal carcinoma of the breast and is positive for both estrogen receptor (ER) and progesterone receptor (PR).

Curcumin (CUR), also known as diferuloylmethane, has the chemical formula 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. It is an active polyphenolic compound that is isolated from the spice turmeric (*Curcuma longa*). Its chemical structure includes α,β unsaturated β -diketones, along with hydrophobic phenyl groups that are vital to its binding activity with molecular targets. While CUR is commonly used as a spice, food additive, and dietary pigment, it has captivated attention for having antibacterial, antiviral, anti-inflammatory, and anticancer properties.^{4–6} It is also considered to have valuable potential with regards to the targeting of various cancers.⁶

Correspondence: Sumi Dinda
Biomedical Diagnostic and Therapeutic
Sciences, 3164 HHB, School of Health
Sciences, 433 Meadow Brook Road,
Oakland University, Rochester,
MI 48309, USA
Tel +1 248 364 8676
Fax +1 248 364 8657
Email sdinda@oakland.edu

CUR also exhibits anticancer effects in cancer stem-like cells (CSCs). It has been shown to considerably diminish microtentacles of the plasma membrane of CSCs, thereby preventing reattachment of the cells at distant tissues.⁷ It has also been demonstrated that CUR restores expression of the tumor suppressor gene E-cadherin in breast CSCs.⁸

A significant number of other reports have indicated that the anticancer effects of CUR are due to the regulation of multiple molecular targets involved in tumorigenesis, angiogenesis, metastasis, invasion, proliferation, and apoptosis. It is also known to significantly reduce the harmful reactions of reactive oxygen species.^{9–23} These anticancer effects are also demonstrated when CUR is present in different analogs, or when combined with other compounds.^{24–32} CUR-loaded nanoparticles may also have potential applications in breast cancer therapy and other various cancers.^{33,34}

Numerous studies have demonstrated that CUR exhibits multiple effects in the suppression of human breast cancer cells *in vitro*, leading to the incorporation of CUR in ongoing clinical trials.^{35–39} It has been reported that CUR hinders the growth of T-47D breast cancer cells in a concentration-dependent manner.⁴⁰

CUR-induced apoptosis has been associated with stimulation of the p53 pathway.⁴¹ Consistent with our findings that CUR decreases the regulation of p53 in T-47D breast cancer cells, it has been demonstrated that CUR impairs the expression and function of p53 in colon cancer cells through inhibition of its gene transcription.⁴² Literature has shown that the p53 gene is mutated in the T-47D breast cancer cell line. However, the actual quantity of mutated versus wild-type p53 remains unknown in the T-47D cells.^{43–45} Studies have also shown that in estrogen receptor (ER)-positive breast cancer cells, the antiproliferative effects of CUR are dependent on the presence of estrogen.^{11,46} Reports indicate that breast cancer cells treated with CUR show a significant downregulation in the expression of ER α compared with the control cells.⁴⁰ Previous studies, including studies from our laboratory, have shown that treatment with estrogen in T-47D breast cancer cells causes increased cell proliferation and upregulation of the tumor suppressor protein, p53.^{47–49} In this study, we have analyzed the effects of CUR on the expression of ER α and p53 in the presence of estrogen, bisphenol-a (BPA), and anti-estrogens in T-47D breast cancer cells. Our studies validate CUR's potential as a therapeutic agent for breast cancer.

Methods and materials

Cell culture and treatment with ligands

T-47D human breast cancer cells (ATCC[®] HTB-133[™]; American Type Culture Collection, Rockville, MD, USA)

were routinely cultured in RPMI-1640 media (Hyclone, Logan, UT, USA) containing 2 mM l-glutamine, 25 mM HEPES, 24 mM sodium bicarbonate, 0.5% 100 \times non-essential amino acids, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 mg/mL amphotericin B (Hyclone), and 0.14 IU/mL insulin (Sigma, St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone). In all studies, cells were plated in 10% FBS media and incubated at 37°C in the presence of 5% CO₂. After the cells were allowed to attach and grow for 48 hours, they were cultured in 5% FBS stripped with dextran-coated charcoal (DCC-FBS), which ensures steroid-free treatment conditions, for a total of 6 days with fresh medium added every 48 hours. On the sixth day, semi-confluent cells were treated with various ligands for 3 or 24 hours. Stock solutions of the ligand used for cell treatment were prepared in DMSO to a 1000-fold higher concentration than the final concentration to be extracted.

Extraction of cells

Following the removal of media by aspiration, cells were washed with ice-cold Hanks balanced salt solution and scraped into 200 μ L of RIPA lysis buffer. The cells were thawed on ice. To prepare a high-speed supernatant (HSS), the cells were centrifuged for 15 minutes at 15,000 \times g at 4°C. The HSS for each sample was stored at –80°C until further use.

SDS–PAGE and Western blot analysis

Extracted protein supernatants were quantified using the Bradford method to standardize the amount of protein to be loaded into each lane. After the supernatants for each sample were denatured for 3 minutes at 85°C, 30 μ g aliquots of total protein were loaded into each lane on a 7.5% polyacrylamide gel. The gels were subjected to electrophoresis to perform protein band separation. Proteins were wet transferred onto an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) in a tris-glycine buffer system (0.025% SDS and 15% methanol) contained in a Bio-Rad (Hercules, CA, USA) trans blot cell set to 110 V for 45 minutes. Membranes were blocked for 1 hour in 5% (w/v) non-fat dry milk (NFDM) in TBS-T (20 mM Tris–HCl, 140 mM NaCl, pH 7.4, 0.1% [v/v] Tween 20) and incubated with primary antibody anti-ER α clone F-10 and anti-p53 (Santa Cruz) diluted 1:500 in 5% (w/v) NFDM in TBS-T for 2 hours. Actin bands were probed by anti-actin (monoclonal antibody clone C4) (Millipore). ER α and p53 levels were normalized to protein levels of the evolutionarily conserved actin protein according to the manufacturer's protocol. After washing with TBS-T (3 \times 10 minutes) and incubation with horse radish peroxidase (HRP)-conjugated goat antimouse

IgG_{2a} secondary antibody (Santa Cruz) diluted 1:1000 in 5% (w/v) NFD in TBS-T for 1 hour, blots were developed using Amersham ECL Prime (GE Healthcare Biosciences, Piscataway, NJ, USA). ER α and p53-specific bands were visualized using the Bio-Rad Chemi-Doc XRS + System. After immunoblotting, the PVDF membranes were stained with Coomassie blue to ensure the correct normalization against total protein levels and full transfer of protein. The protein band density on each membrane was quantified using the Image Studio Lite program version 3.1 (LI-COR Biosciences, Lincoln, NE, USA).

Cell viability and apoptosis assays

All growth studies were conducted in 12-well culture plates. To ensure active, non-confluent cell populations during treatment duration, 12-well culture plates were initially seeded with 3.0×10^4 cells per well in 1 mL culture medium containing 10% FBS. Studies were limited to 7-day total duration to correlate with the results of Western blot analyses. On the seventh day, the cells were trypsinized and removed from individual wells of the culture plate. Cells were stained with propidium iodide (PI) for viability assays and fluorescein isothiocyanate-conjugated Annexin V for apoptosis assays per manufacturer's instruction (Cellometer Inc., Lawrence, MA, USA). The cells underwent imaging cytometry by the Cellometer Vision CBA and FCS Express-6 software. The fluorescent threshold in the software was set to 0% to measure total fluorescence of each counted cell from the captured images. The measured fluorescence intensities were exported to Microsoft Excel and converted to FCS file for analysis in the De Novo Software.⁵⁵

Immunofluorescence and confocal microscopy

T-47D cells were plated on cover slips in 12-well plates (30,000 cells/well) and cultured for 48 hours in a 10% FBS medium. The medium was then changed to 5% DCC-FBS, and fresh medium was added at 2-day intervals. The cells were cultured in this medium for a total of 4 days. On the fourth day, the ligands were suspended in 5% DCC-FBS media, and semi-confluent cells were treated for 24 hours.

Cellular localization of p53 by immunocytochemistry

The cells were fixed on cover slips for 10 minutes with 1% formalin in phosphate-buffered saline (PBS), permeabilized with ice-cold acetone and methanol (50:50), and washed three times with PBS. Staining procedures were

performed in a humidified chamber at 23°C. Cells were incubated in 10% goat serum (Sigma) to suppress non-specific binding of IgG, followed by 3 hours incubation with 1:150 dilution of anti-p53 (F-10) monoclonal antibody. After washing with PBS, cells were incubated for 3 hours with 1:200 dilution of antimouse IgG conjugated with Cy3 (Jackson Immuno Research Laboratories, West Grove, PA, USA). Cover slips were washed in PBS and incubated for 2 minutes in 1 μ g/mL DAPI dissolved in PBS. Cells were washed three times in PBS, mounted with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA) and stored in the dark at 4°C.

Structural analysis by DIC

DIC images were acquired by using the DIC objectives with Nikon Digital Eclipse C1 Plus confocal microscope. The distribution of three-dimensional fluorescent structures was analyzed using a Nikon Digital Eclipse C1 Plus confocal microscope. NIS elements software (Nikon Instruments, Melville, NY, USA) was used for noise reduction and three-dimensional reconstruction of the images.

Statistical analyses

The results are expressed as mean \pm SEM. Statistical significance was determined by Kruskal–Wallis test followed by post-hoc analysis using Mann–Whitney *U* test. The *p* values were adjusted for multiple testing corrections using the false discovery rate. Differences are considered significant at *p*<0.05. Statistical analyses were carried out using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Effects of CUR on cellular viability and the regulation of ER α and p53 levels: concentration dependency

Figure 1 demonstrates the concentration-dependent effects of CUR on cell viability and the expression of ER α and p53 protein levels. To examine the effects on cell viability, the T-47D cells were treated with 5–100 μ M CUR for 6 days. Cellular viability was then determined by propidium iodide (PI) staining and image cytometry. As shown in Figure 1A, 20–100 μ M CUR significantly decreases T-47D cell viability as compared with the control. CUR at lower concentrations (5–10 μ M) did not display a significant effect on the cell viability compared with the control.

T-47D cells were treated with 5–80 μ M CUR for a duration of 24 hours. The cells were then subjected to

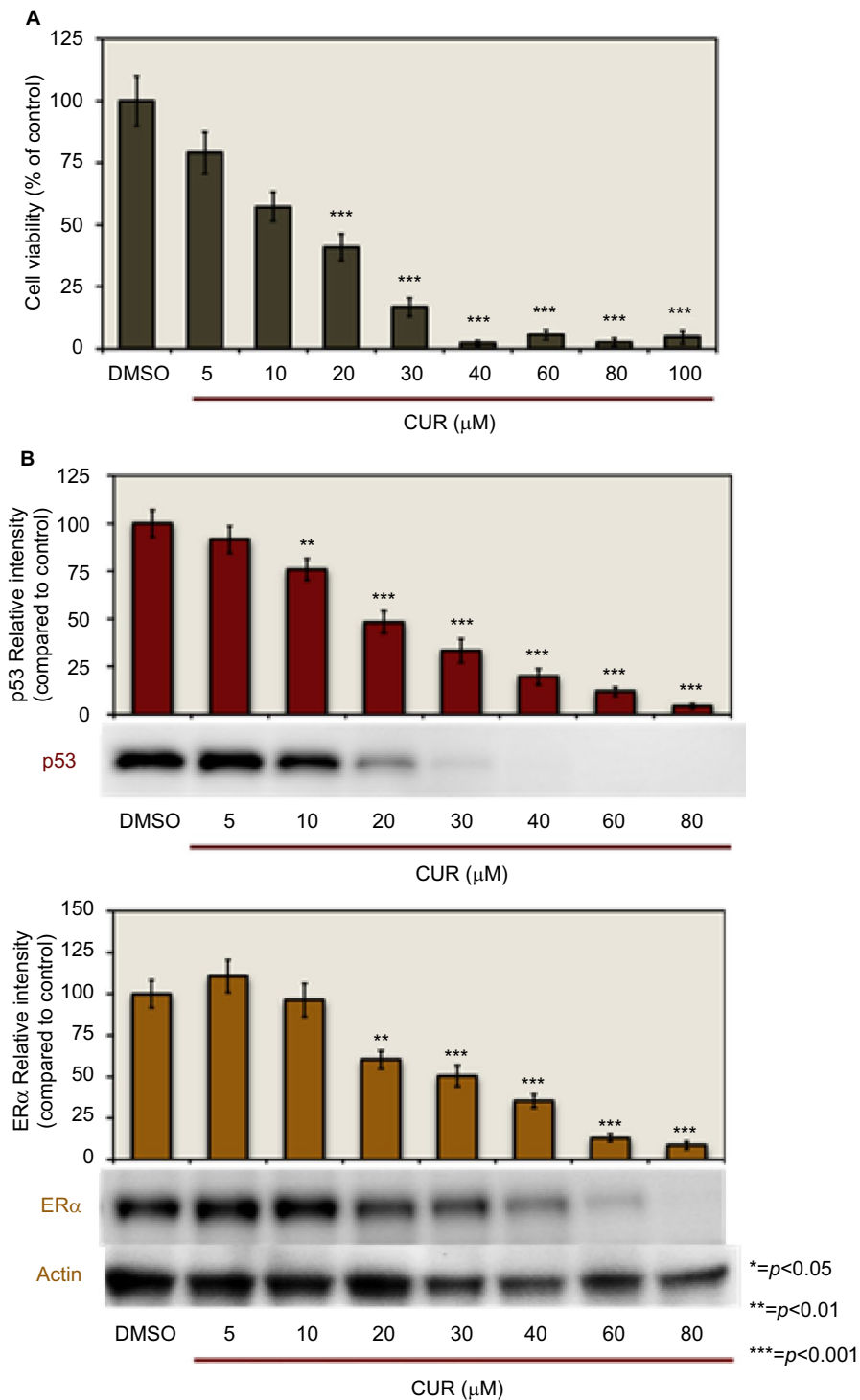


Figure 1 Concentration-dependent effects of CUR: curcumin decreases T-47D cell viability and downregulates p53 and ER α protein expression.

Notes: (A) T-47D cells were treated for 6 days with 5–100 μ M curcumin, and cell viability was determined by PI staining and image cytometry. (B) T-47D cells were treated for 24 hours with 5–80 μ M curcumin and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis. The relative intensities of each band were compared with vehicle control (DMSO). Representative blots from three independent experiments are shown.

Abbreviations: CUR, curcumin; DMSO, dimethyl sulfoxide; ER α , estrogen receptor; PI, propidium iodide.

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis to examine the concentration-dependent effects of CUR on the level of ER α and p53 (Figure 1B). The relative densities of each

of the protein bands were compared with the control. Upon examination of each of the densities, Figure 1B shows that 20–80 μ M CUR significantly downregulates both ER α and p53 protein levels.

Differential interference contrast microscopy of 60 μ M CUR cytotoxicity in T-47D cells

Differential interference contrast (DIC) images were taken of the T-47D cells to see the contour of the cells (Figure 2A). T-47D cells were treated with 60 μ M CUR for 6 days, and images were acquired as described in Methods and Materials section. The merged images in Figure 2A indicate that 60 μ M CUR depleted the T-47D cells.

Effects induced by 60 μ M CUR in combination with MG-132

MG-132 is a proteasomal inhibitor that is known to prevent the degradation of short-lived proteins. Therefore, it is a valuable research tool for examining the cellular degradation through the proteasome pathway.^{50,51} To determine whether CUR is an inhibitor of proteasomal degradation, 60 μ M CUR was analyzed to verify if MG-132 reversed its effects on the downregulation of protein levels (Figure 2B).

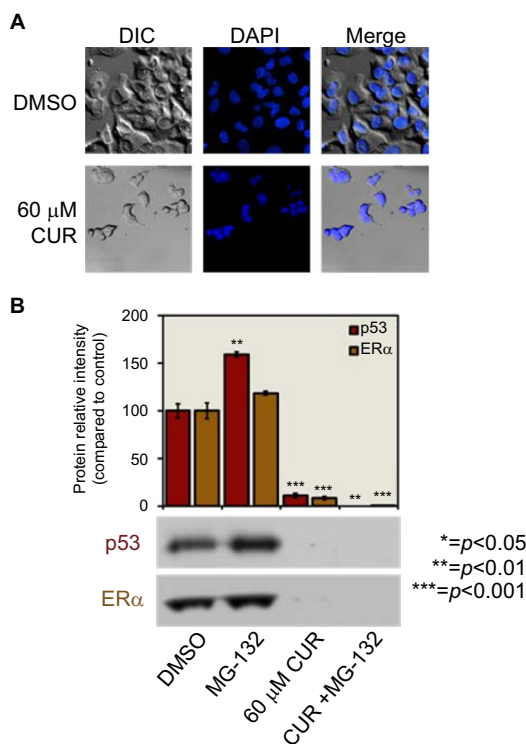


Figure 2 Effects induced by 60 μ M CUR.

Notes: (A) Differential interference contrast microscopy of 60 μ M curcumin cytotoxicity in T-47D cells. T-47D cells were treated for 6 days with 60 μ M curcumin, and images were acquired by Cellometer Vision CBA and FCS Express-6 software. (B) Downregulation of p53 and ER α by 60 μ M curcumin is not reversed by the proteasomal inhibitor MG-132. T-47D cells were pretreated for 30 minutes with 10 μ M MG-132 followed by 3 hours treatment with 60 μ M curcumin. Protein extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis, and the relative intensities of each band were compared with vehicle control (DMSO). Representative blots from three independent experiments are shown.

Abbreviations: CUR, curcumin; ER α , estrogen receptor; DIC, Direct Image Contrast; DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

T-47D cells were pretreated with 10 μ M MG-132 for 30 minutes, followed by a 3-hour treatment with 60 μ M CUR. Protein extracts were subjected to SDS–PAGE and Western blot analysis, and the relative densities of each band were compared with control (dimethyl sulfoxide [DMSO]), as described in Methods and Materials section. As indicated in Figure 2B, MG-132 did not reverse the effects of 60 μ M CUR, which suggests that the downregulation of protein levels caused by CUR is not due to mechanistic causes but rather due to its cytotoxicity to the cells.

Effects of 20 and 60 μ M CUR on the apoptosis of T-47D cells

As illustrated in Figure 3, scatter plots following Cellometer Vision CBA and FCS Express-6 Analysis show an increased trend in the apoptotic/necrotic cell population following incubation with 60 μ M of CUR treatment for 6 hours compared with the negative controls. This trend explains a shift from the apoptotic (annexin V-positive only) to necrotic (annexin V and PI positive) cell population. This trend seems to increase when the cells were treated with 60 μ M of CUR for 24 hours. However, treating the cells with 20 μ M of CUR has shown a lesser trend of apoptotic/necrotic cell population in 6 and 24 hours.^{52–55}

Effects of CUR on the immunolocalization of p53 in T-47D cells

Along with Western blot studies (Figures 1B and 5B), image analysis was performed by confocal microscopy involving immunofluorescence to determine the cytolocalization of cel-

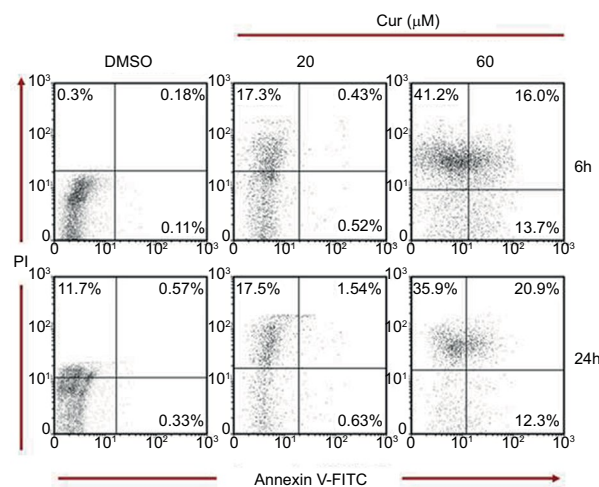


Figure 3 Apoptosis assay: effects of 20 and 60 μ M curcumin on T-47D cells.

Notes: T-47D cells were treated with 20 and 60 μ M curcumin for 6 or 24 hours and analyzed for Annexin V-PI positivity by image cytometry which were acquired by Cellometer Vision CBA and FCS Express-6 software.

Abbreviations: CUR, curcumin; DMSO, dimethyl sulfoxide; PI, propidium iodide.

lular p53. 4',6-Diamidino-2-phenylindole (DAPI) is used to stain the nuclei of the cells so that the nuclei will be displayed in the image analysis (Figure 4). As shown in Figure 4, p53 is localized exclusively in the nuclei of the T-47D cells. The control group (DMSO) represents cells grown in a charcoal-treated serum that displays immunofluorescence localized in the nuclei of the T-47D cells. Treatments of 20 μM and 60 μM CUR did not affect the cytolocalization of cellular p53, as the image analysis shows p53 to still be localized in the nucleus (Figure 4). The images of the cells stained for the nuclei and stained for p53 were merged to further demonstrate that p53 is localized exclusively in the nuclei.

Effects of CUR in combination with ER agonists and antagonists on cellular viability and expression of ER α and p53

Figure 5 displays the effects of ER agonists and antagonists in combination with CUR. T-47D cells were treated with 20 and 60 μM CUR, 10 nM E₂, 600 nM BPA, 1 μM ICI, and 1 μM tamoxifen (TAM) either alone or in combination. As mentioned in Methods and Materials section, the cells studied for cell viability were treated for 6 hours, and the cells being studied for p53 and ER α expression by Western blot analysis were treated for 24 hours. The data show that even with the addition of the anti-estrogens ICI and TAM, the estrogen E₂, and the ER agonist BPA, the effect of CUR on T-47D cell viability could not be reversed (Figure 5A).

Figure 5B indicates that when the cells were treated with 20 and 60 μM CUR, the level of p53 protein expression was significantly downregulated compared with the control. The

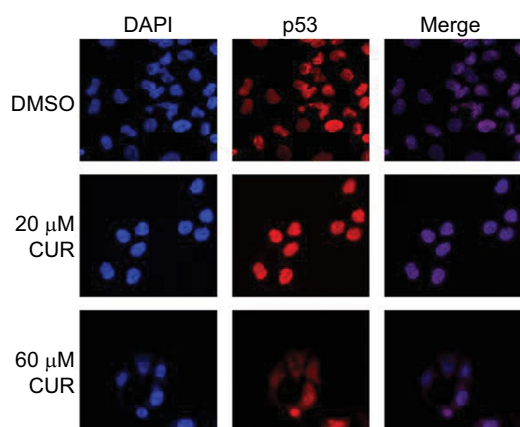


Figure 4 Effects of CUR on the immunolocalization of p53 in T-47D cells.
Notes: Image analysis was performed by confocal microscopy involving immunofluorescence to determine the cytolocalization of cellular p53. Treatments of 20 and 60 μM CUR did not affect the cytolocalization of cellular p53. The images of the cells stained for the nuclei and stained for p53 were merged to further demonstrate that p53 is localized exclusively in the nuclei.
Abbreviations: CUR, curcumin; DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

addition of E₂, ICI, and TAM to the treatment of 20 μM CUR did not alter the significant downregulation of p53, but the addition of BPA with 20 μM CUR had no effect. The additions of E₂, BPA, ICI, and TAM to the 60 μM treatment of CUR still resulted in the downregulation of p53.

As shown in Figure 5B, the expression of ER α is also significantly downregulated compared with the control by 20 and 60 μM CUR. It is also demonstrated that the addition of E₂, BPA, ICI, and TAM to both 20 and 60 μM CUR did not reverse the downregulation of ER α expression (Figure 5B).

Discussion

Our studies demonstrate that 20–80 μM CUR significantly downregulates both p53 and ER α protein levels with a concomitant decrease in T-47D cell viability. Other studies involving different breast cancer cell lines treated with CUR, such as BT-483 and MDA-MB-23, have shown a significant decrease in cell proliferation. For example, 48-hour treatments of 5 $\mu\text{L/mL}$ CUR inhibited proliferation of BT-483 cells by 60%–70% and MDA-MB-23 cells by 50%–60%.⁵⁶ CUR has been found to be highly cytotoxic to some breast cancer cell lines. This is consistent with our findings that the proteasomal inhibitor MG-132 did not reverse the effects of 60 μM CUR on the downregulation of ER α and p53 in T-47D cells.⁵⁷ In our studies, the presence of anti-estrogens and ER agonists, including BPA, did not reverse the effects of CUR on T-47D cell viability, and this reduction could not be explained by an induction of apoptosis under our experimental conditions. In addition, when CUR is used in combination with ER α agonists and antagonists, the alterations in p53 protein expression do not correlate with alterations in cell viability.

It has been shown that CUR inhibits the phosphorylation of p53, its ability to bind to DNA, and the transactivation of genes that correlate with its function as a tumor suppressor.⁴² However, our experiments suggest that the CUR-induced effects on the T-47D cells are not due to a mechanism involving p53 and appear to involve a mechanism that includes proteins other than p53 and ER α . CUR has also been found to inhibit the expression of the leptin gene in T-47D breast cancer cells, and this inhibition was positively associated with a decrease in the expression of ER α .⁴⁰ Experiments involving the multiple effects of CUR on human breast cancer cells have shown that the antiproliferative effects of CUR are estrogen-dependent in ER-positive MCF-7 breast cancer cells.⁴⁶ These findings suggest that CUR acts through estrogen receptors to demonstrate its anticancer effects in hormone-dependent breast cancer cells. However, our results indicate that CUR-induced effects are not mediated via ER α . These results are supported

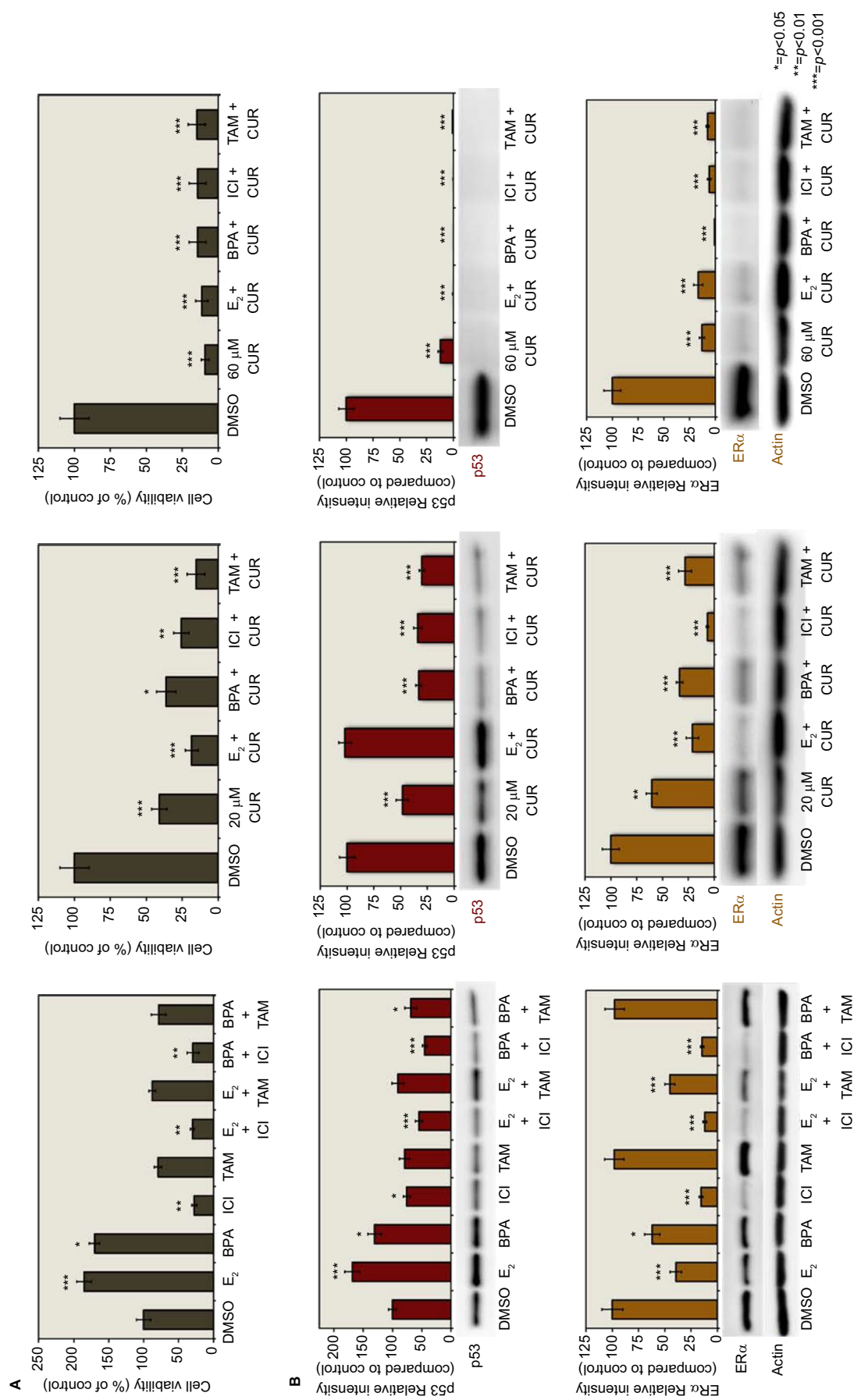


Figure 5 Curcumin-induced effects are not mediated by ER α .
Notes: T-47D cells were treated with 20 and 60 μ M curcumin, 10 nM E₂, 600 nM BPA, 1 μ M ICI, and 1 μ M TAM either alone or in combination for (A) 6 days or (B) 24 hours. (A) Cell viability was determined by propidium iodide staining and image cytometry. (B) Western blot analysis. The relative intensities of each band were compared with vehicle control. dimethyl sulfoxide, curcumin; BPA, Bisphenol A.
Abbreviations: DMSO, dimethyl sulfoxide; TAM, tamoxifen; CUR, curcumin; BPA, Bisphenol A.

by the findings of CUR having antiproliferative effects on the ER-negative MDA-MB-231 breast cancer cell line, and these effects do not depend on the presence of estrogen.⁴⁶ When MDA-MB-231 cells are treated with a combination of CUR and epigallocatechin gallate, another polyphenolic compound, antiproliferative effects are still present, further suggesting that the effects of CUR do not involve ER α .²⁶

It remains clear that 20–80 μ M CUR affects p53 and ER α protein expression on a molecular level. Whether these two proteins, known to contribute to the formation and progression of hormone-dependent breast cancer, are regulated on a transcriptional or translational level remains unclear. Although several studies have indicated estrogen-dependent effects of CUR, our results demonstrate effects that are not dependent on the presence of estrogen. Therefore, the effectiveness of CUR on hormone-dependent breast cancer cells needs to be re-evaluated. CUR has been studied as a chemopreventive agent in numerous clinical trials, and its effects should be further examined in vivo as well as in vitro.^{35–39} Further studies are warranted as our results support the potential and suitability of CUR as a treatment modality in hormone-dependent breast cancer.

Acknowledgment

Amy E Siebert is currently working in Department of Biological Sciences at Oakland University.

Disclosure

The authors report no conflicts of interest in this work.

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