# Curcumin inhibits the expression of ornithine decarboxylase and adenosine deaminase genes in MCF-7 human breast cancer cells

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**Abstract:** Curcumin is the active ingredient of *Curcuma longa*, which inhibits the development of malignant cells. Prevention and treatment of cancer by natural compounds, especially curcumin, and understanding the mechanism of action, is an area of interest in cancer research. In this study, we evaluated the effects of curcumin on cell proliferation, ornithine decarboxylase 1 (ODC1) and adenosine deaminase (ADA) gene expression in human breast cancer cell line (MCF-7) as compared to the non-cancer line (MCF-10A). Both cell lines were subjected to increasing doses of curcumin, ranging from 0 to 30 μg/mL. Cell viability was quantified by the MTT assay. *In vitro* clonogenic survival assay was performed on MCF-7 cells. Expression of ADA and ODC1 were analyzed by Western blotting and qRT-PCR. Curcumin inhibited the growth of malignant cells in a time- and dose-dependent manner. The calculated $IC_{50}$ value for MCF-7 cells in 48 h was 12 μg/mL. Forty-five to 70% decreases in colony formation were observed in MCF-7 cells treated with 30-60 μg/mL curcumin, respectively. Our data revealed a dose-dependent downregulation of ODC1 and ADA expression and respective enzyme activities by curcumin, which correlated with decreased proliferation in the MCF-7 breast cancer cell line. These data suggest that curcumin represses the proliferation of breast cancer cells through downregulation of ODC1 and ADA gene expression, which might be another mechanism of curcumin-mediated tumor growth inhibition.

**Key words:** antitumor; breast cancer; cell proliferation; adenosine deaminase (ADA); ornithine decarboxylase 1 (ODC1)

## INTRODUCTION

Breast cancer is the most common cancer in developed and developing countries and is in fifth place in all types of cancers. In women, breast cancer is the second leading cause of death after lung cancer. In America, one out of every eight women has the potential to develop breast cancer [1]. In Asia, there is an increase in the incidence of breast cancer due to lifestyle changes. Mortality rates are rising due to the lack of screening programs and therapeutic facilities for breast cancer in developing countries. [2]. Therefore, providing counseling for preventing this type of cancer is of great importance in these communities. In this regard, the use of diets containing natural compounds that are commonly found in traditional medicine in these societies and have general acceptance could be a suitable approach in preventing and treating breast cancer.

Curcumin, as a natural compound derived from *Curcuma longa*, is used in traditional medicine and diet in Asian countries, including Iran [3]. Curcumin is effective in the treatment of cardiovascular, pulmonary and metabolic diseases as well as diseases of the nervous system [4]. Several laboratories and clinical studies have indicated the role of curcumin in the prevention and treatment of various types of cancer [5]. In studies of the antitumor effects of curcumin, several mechanisms have been proposed and proven, including apoptosis [6] and cell cycle arrest [7].

ODC1 and ADA are two important enzymes in cell proliferation and differentiation and are also effective in tumor progression [8,9]. ODC1 is the first enzyme in the biosynthesis pathway of polyamines, molecules that seem to support the proliferation and expansion of cancer cells by inhibiting apoptosis [10]. Zhu et al. [11] reported increased ODC1 activity and polyamine

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639

concentrations in breast cancer tissue as compared to normal breast tissue, and that polyamine biosynthesis inhibitors decreased the growth of cancerous tumors. This enzyme was investigated in curcumin-related studies at the level of enzymatic activity. ADA, which assumes a role in purine metabolism, converts the molecule of adenosine into inosine [12]. According to previous studies, the enzyme activity of ADA is increased in human breast cancer tumors in contrast with normal tissues [8,13]. In cancer cells and tissues, high ADA activity is responsible for the reduction of adenosine, a molecule that inhibits the growth of cancer cells by antiinflammatory responses [8]. Therefore, ADA can be a new target for curcumin. In the present study, the antiproliferative activity of curcumin as a result of reduced expression of ODC1 and ADA genes in human breast cancer cells is evaluated.

## MATERIALS AND METHODS

### Materials

MCF-7 and MCF-10A cells were obtained from the Pasteur Institute (Tehran, Iran). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, and incubated at 37ºC in a humidified incubator containing 5% $CO_2$. Curcumin was purchased from Sigma (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) to a 10-mM stock solution, and stored at -20°C.

### Cell proliferation assay

The cytotoxic activity of curcumin was determined by the MTT assay. Breast and normal ($4 \times 10^3$) cells in 200 µL DMEM were seeded in a sterile 96-well plate and incubated for 24 h at 37°C. The cells were treated with different concentrations of curcumin (0, 10, 20 and 30 µg/mL) and were incubated for 24, 48 and 72 h. A 2-mM MTT solution was added to each well, followed by 4 h of incubation. After removing the supernatant, 100 mL of DMSO was added and the plate was shaken for 10 min. Finally, the optical density was determined at 540 nm using an ELISA microplate reader (Awareness, Palm City, FL, USA). In this assay Paclitaxel (Sigma), a known cytotoxic agent, was used as a positive control.

### Colony formation assay

Cells (500 cells/mL) were plated in 60-mm Petri dishes and allowed to adhere for 24 h. The next day, the cells were treated for 48 h with curcumin (10, 20 and 30 µg/mL), or 0.1% DMSO. The colonies were fixed with a solution of acetic acid and methanol (1:3) for 15 min, stained with 0.5% crystal violet for 30 min and counted under the stereomicroscope [14].

### Ornithine decarboxylase (EC 4.1.1.17) and adenosine deaminase (EC 3.5.4.4) activity assays

ODC1 activity was assayed by determination of $^{14}CO_2$ formation as previously described [15]. ADA activity was determined by the colorimetric method of Giusti [16]. The results are presented as percent inhibition obtained after comparison to the activity of the controls.

### Western blot analysis

Total proteins extraction and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously [17]. Briefly, the cells were lysed with a cell lysis buffer for 30 min to 1 h on ice. The cell lysates were centrifuged at 7,000$xg$ for 10 min at 4°C and the concentration of proteins was determined by the Bio-Rad Protein Assay Reagent. The proteins were subjected to 12% SDS-PAGE. Following electrophoresis, the gels were transferred to polyvinylidene difluoride membrane. After the blocking step, blots were exposed to the primary and secondary antibodies. Detection was performed using chemiluminescence detection reagents, and imaging was carried out using a chemiluminescence analyzer (Bio-Rad Laboratories). Quantification was performed by ImageJ software.

### qRT-PCR

The cells were cultured in three groups and treated with increasing concentrations (0-60 µg/mL) of curcumin for 48 h. Total cellular RNA was extracted from the cells using the Easy BLUE Total RNA Extraction kit (iNtRON Biotechnology, Seoul, Korea). Two µg of RNA was reverse transcribed into cDNA with a reverse transcription reaction mixture using the Power cDNA Synthesis kit (iNtRON). The cDNA

was then amplified and quantified using the SYBR Green PCR kit (Takara Bio, Japan) with the ABI Step-One system (Applied Biosystems, Foster City, CA, USA). Primers for ODC1, ADA and βACT were designed using Oligo 7.5 software (Molecular Biology Insights Inc., Colorado Springs, USA). The primer sequences for ODC1 were: FW: 5´GTGGGTGATT-GGATGCTCTTTG 3´ RV: 5´AGGCCCTGACAT-CACATAGTAG 3´. The primer sequences for ADA were: FW: 5´ACCAGGCTAACTACTCGCTCAA3´ RV: 5´TCAGTAAAGCCCATGTCCCGTT3´. Primer sequences for βACT were: FW: 5´TCCATCAT-GAAGTGTGACGT3´ RV: 5´GAGCAATGATCTT-GATCTTCAT3´.

A PCR reaction mixture of 20 μL contained 10 μL SYBR Green Master Mix, 0.4 μL reverse primer and 0.4 μl forward primer, 6.2 μl dH2O (RNase free) and 3 μl cDNA (4 ng). Three pairs of primers were used separately. The thermal cycling conditions were as follow: denaturation cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and at 60°C for 60 s (annealing and extension temperature). The qRT-PCR data were analyzed using the relative gene expression ($\Delta\Delta$Ct) method. Briefly, the results are presented as the fold change in gene expression normalized to the reference gene (β-actin) and were determined using the equation fold change=$2^{-\Delta\Delta Ct}$ [18]. For each single reaction, melting curve analysis was performed to exclude amplification of nonspecific products. Each valid amplification reaction showed a single peak at the desired melting temperature.

## Statistical analysis

The data are presented as the mean±SEM. Student's t-test was used and P<0.05 was taken as the level of significance. All results were analyzed by statistical software SAS (USA).

## RESULTS

### Cytotoxicity of curcumin

The MTT and clonogenic assays were conducted to evaluate the potential cytotoxic effect of curcumin on human breast cancer cells (MCF-7) and human mammary epithelial cells (MCF-10A). The cell lines were treated with different concentrations of curcumin for 24, 48 and 72 h, and cell viability was measured by the MTT assay. The results demonstrated that curcumin significantly decreased the viability of malignant cells in a time- and dose-dependent manner. This antiproliferation effect was observed within a 24 h period, and continued to increase over the next 72 h. The survival rates of MCF-7 cells were 62, 36 and 22% after exposure to 10, 20 and 30 μg/mL curcumin for 48 h, respectively (Fig. 1B); they were lower than those for MCF-10A cells (Fig. 1A). Curcumin IC$_{50}$ values of MCF-7 cells were 12 and 7.5 μg/mL for 48 and 72 h, respectively. Therefore, nontoxic concentrations (<10 μg/mL for 48 h) of curcumin were used in subsequent experiments. Colony formation in MCF-7 cells after curcumin treatments significantly decreased in a dose-dependent manner.
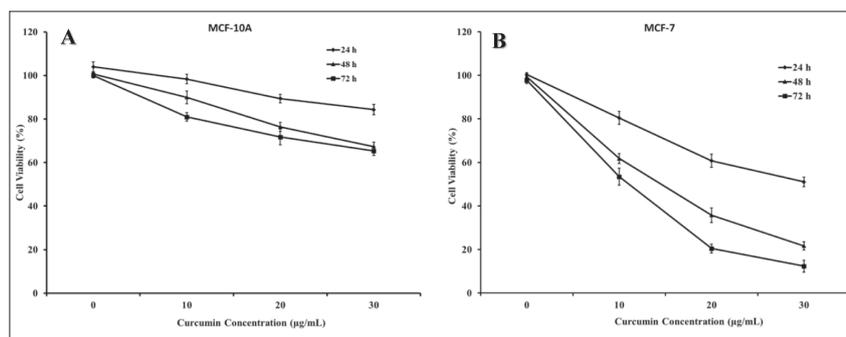


**Fig. 1.** Cytotoxic effects of curcumin on MCF-10A (**A**) and MCF-7 (**B**) human breast cell lines. Cells were treated for 24, 48 and 72 h with different concentrations of curcumin. Cytotoxicity was determined by the MTT assay. Values represent means±SEM.
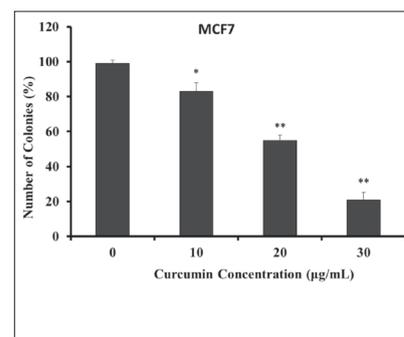
**Fig. 2.** Colony formation assay shows a significant reduction in colony formation in MCF-7 cells treated with different concentration of curcumin. Values represent means±SEM, *p<0.05 and **p<0.01 compared to control.
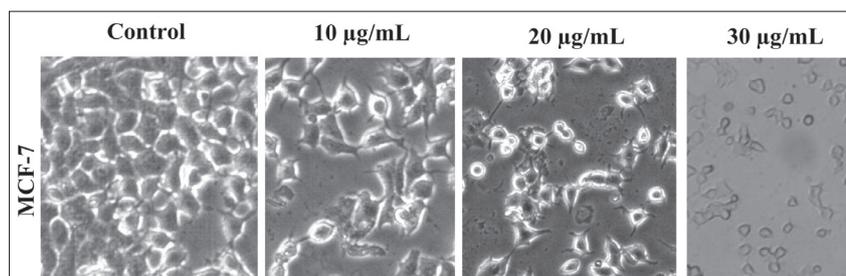
**Fig. 3.** Morphological changes of MCF-7 cells treated with different concentrations of curcumin for 48 h viewed under the inverted phase-contrast microscope (×200). A reduction in cell population was noted with the increase in the concentration of the treatment as compared to control (untreated cells).
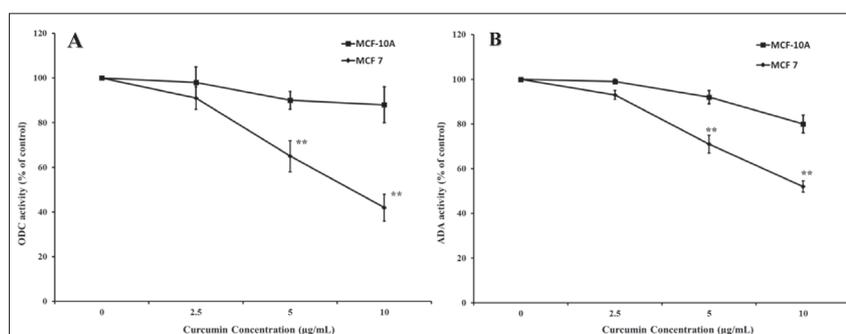


**Fig. 4.** The effect of curcumin on ODC1 (**A**) and ADA (**B**) activities. The cells were treated with the indicated concentrations of curcumin for 48 h. The cells were harvested and ODC1 and ADA activities were determined. The effects of the treatment are expressed as the percentage of enzyme activities in control cells. Values represent the means±SEM, *p<0.05 and **p<0.01 compared to the control.

Thirty μg/mL and 60 μg/mL curcumin caused 45 and 80% decreases in colony formation in MCF-7 cells, respectively (Fig. 2). Cytotoxicity results were correlated with morphological alterations (loss of cell volume, cell shrinkage and nuclear condensation). Cells treated with high concentration of curcumin became spherical and shrunken, while untreated cells remained normal in size and shape (Fig. 3). Paclitaxel as a positive control at a 700 nM concentration decreased the viability of MCF-7 cells by 6.8±0.3% after 48 h in comparison with untreated control cells.

### ODC1 and ADA activities

To determine how regulation of ODC1 and ADA enzyme activities was involved in the antiproliferative effect of curcumin, MCF-7 and MCF-10A cells were treated with concentrations of curcumin ranging from 0 to 10 μg/mL for 48 h. The effect of the treatment of cells with curcumin on ODC1 and ADA activity are shown in Fig. 4. Treatment with 10 μg/mL curcumin

resulted in 5.5- and 4-fold decreases in ODC1 and ADA activities as compared to control cells, respectively. The treatment of cells with curcumin resulted in significant dose-dependent inhibition of enzyme activities.

### Changes in ODC1 and ADA expression

In order to investigate the level of ODC1 and ADA regulation (translational or transcriptional) by curcumin, immunoblotting and qRT-PCR assays were performed to assess the expression of ODC1 and ADA in MCF-7 and MCF-10A cells. The results revealed that curcumin significantly downregulated the expression of ODC1 and ADA proteins (Fig. 5) and genes (Figs. 6 and 7) in a dose-dependent manner in the MCF-7 cancer cell line, but not in non-cancer MCF-10A cells. Curcumin at a concentration of 10 μg/mL significantly decreased the expression of ODC1 and ADA genes by 3.5- and 1.9-fold, respectively, in MCF-7 cells. However, in MCF-10A cells, the mRNA levels of ODC1 and ADA were reduced by only 1.3-fold at 10 μg/mL concentration. (Fig. 6-7).

### DISCUSSION

Curcumin is an effective and multitarget natural product for the treatment and prevention of many cancers, including breast cancer [19]. Different studies have examined the mechanism of its activity, and it appears to influence different proteins and genes, such as p53, NF-κB, PI3K-AKT and COX2 [20]. In the present study, we examined a less considered role of curcumin, its impact on two ODC1 and ADA enzymes. The former enzyme is involved in the metabolism of polyamines and the latter in purine metabolism, and the activities of both are significantly increased in malignant cells [8,11]. In this research,
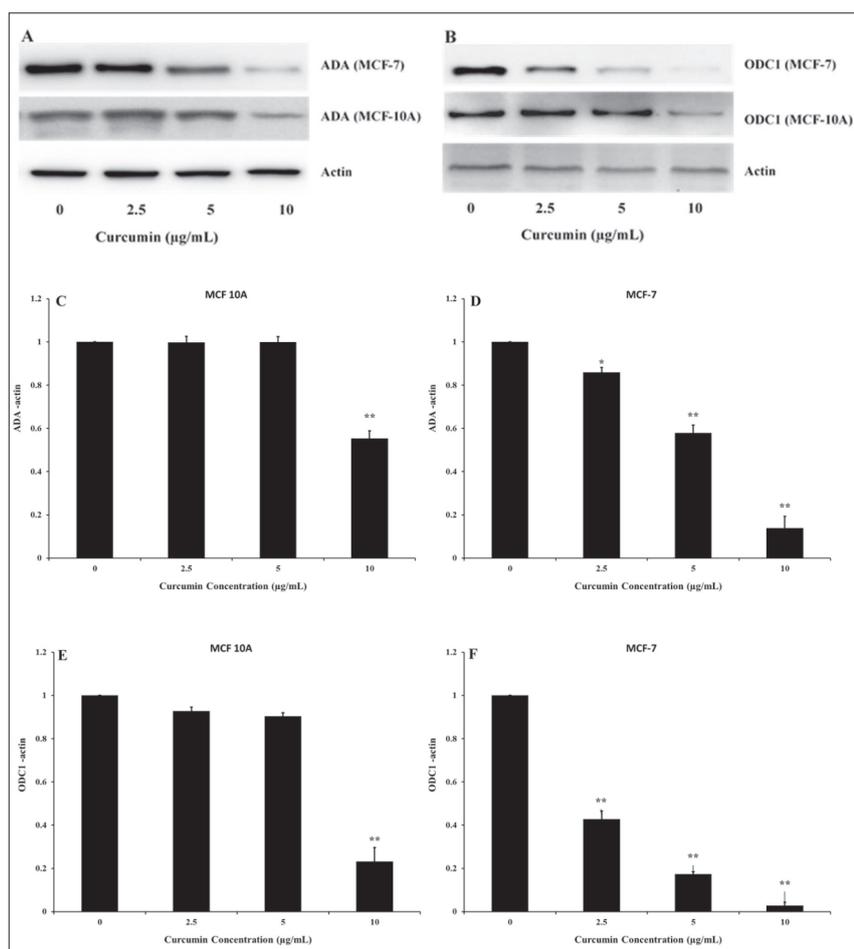
**Fig. 5.** Western blot analysis (**A, B**) and a histogram presenting the expression of adenosine deaminase (**C, D**) and ornithine decarboxylase (**E, F**) in MCF-10A and MCF-7 cells after treatment with 0-10 µg/mL curcumin for 48 h. Values are means±SEM, *p<0.05 and ** p<0.01 compared to control.
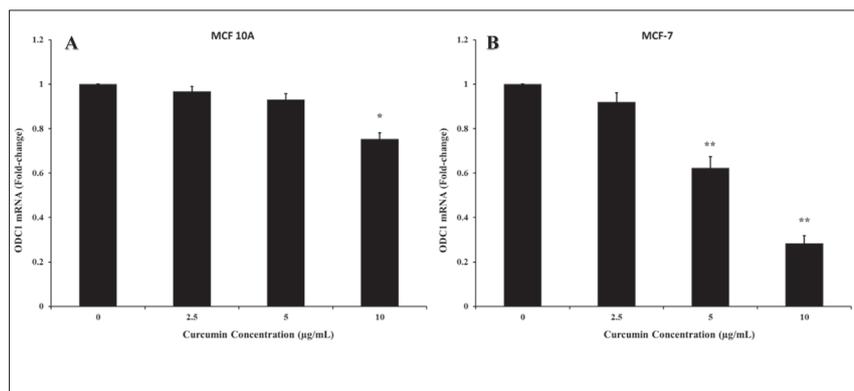


**Fig. 6.** qRT-PCR analysis of ODC1 mRNA expression in MCF-10A normal breast cells (**A**) and MCF-7 cancerous breast cells (**B**). The cells were incubated with the indicated concentrations of curcumin for 48 h. ODC1 mRNA levels decreased in the cancer cell lines in a dose-dependent manner. Relative abundance of mRNA was obtained by normalization to β-actin expression. Values represent means±SEM, *p<0.05 and **p<0.01 compared to control.

the antiproliferative effect of curcumin on the human breast cancer cell line MCF7 was dose- and time-dependent. Likewise, the CFU assay indicated that breast cancer cells after prolonged exposure to curcumin significantly lost the ability to form colonies. Many reports have shown that curcumin and its derivatives have growth inhibitory or antiproliferative effects on cancer cells. It was demonstrated [21] that curcumin inhibits the growth of HL-60 cells by inducing apoptosis. The antiproliferative activity of curcumin was also demonstrated by arresting the cell cycle at the G2/M phase in MCF7 cells [22]. To determine the antiproliferative mechanism of curcumin, the enzymatic activity of ODC1 in normal and malignant cells was measured after exposure to nonlethal concentrations of curcumin, revealing a decline in the activity of this enzyme. ODC1 catalyzes the first and rate-limiting step in the biosynthesis of polyamines and has a very short half-life [23]. Regulation of the activity of this enzyme can also be at the protein level through degradation in the proteasome by an antizyme, or at the transcript level by reducing mRNA production [24]. According to our results, the decrease at the levels of ODC1 protein and mRNA after treatment with curcumin suggests regulation of ODC1 at the transcript level. In parallel with our findings, Berrak et al. [7] showed in curcumin-treated MCF-7 cells that ODC1 expression was downregulated. Reducing the amount of ODC1 protein diminishes the amount of polyamines, which are important molecules that scavenge excess reactive oxygen species (ROS) [25]. Increasing
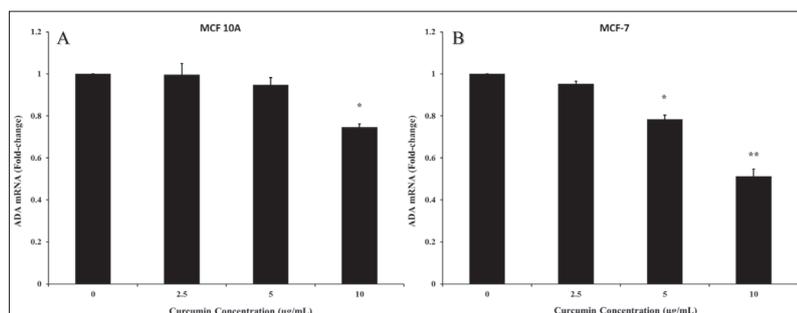
**Fig. 7.** qRT-PCR analysis of ADA mRNA expression in MCF-10A normal breast cells (**A**) and MCF-7 cancerous breast cells (**B**). The cells were incubated with the indicated concentrations of curcumin for 48 h. ADA mRNA levels decreased in the cancer cell line in a dose-dependent manner. The relative abundance of mRNA was obtained by normalization to β-actin expression. Values represent means±SEM, *p<0.05 and **p<0.01 compared to the control.

free radicals leads to the destruction of cellular structures and molecules and ultimately causes apoptosis [26]. Thus, studies have demonstrated that curcumin specifically disables ROS [27]. This is a paradox that curcumin, itself a free-radical scavenger, causes the accumulation of free radicals. Curcumin promotes apoptosis via other pathways, mediated by NF-κB, STAT-3 and PI3-kinase/AKT [28].

Our results showed that the activity and expression levels (protein and mRNA) of ADA in MCF7 cells treated with curcumin declined. The correlation between results of Western blots and qRT-PCR showed that the regulation of ADA protein by curcumin was at the level of transcription. ADA is a protein that, in addition to its importance in the metabolism of purines in the cell, also affects adenosine receptor activity outside the cell, which plays an important role in the physiological activity of the nervous system [29]. Therefore, ADA is important both as an enzyme and allosteric modulator [30]. Cancer cells require purine precursor molecules because of their increased proliferation activity. In addition, reducing the amount of adenosine molecule that plays an important role in antiinflammatory responses is an added advantage [31]. Thus, curcumin diminishes purine precursors by lessening ADA gene expression, and on the other hand, in the absence of adenosine catabolism, the pathway for antiinflammatory responses becomes more active and cells lose their ability to proliferate [32].

In this study, we examined the effects of curcumin on the estrogen receptor-positive cell line (MCF7) (ER+/PR+/HER2-), and our findings regarding the

cytotoxicity and enzymatic activities are similar to other works on the ER/PR/HER2 triple negative breast cancer cell line (MDA-MB231) [33]. Therefore, the antiproliferative effect of curcumin and its inhibitory effect on ODC1 and ADA activities is not estrogen-dependent.

In conclusion, the results of this study indicate that curcumin reduces the expression of ODC1 and ADA genes at the transcriptional level in the human breast cancer MCF7 cell line. Curcumin, in addition to the usual pathways for growth inhibition and apoptosis, seems to trigger antitumor activity by regulating the expression of other genes that are involved in the formation of cancer, including ODC1 and ADA. Further studies on the upstream regulatory elements of these genes will provide a better understanding of the mechanism of curcumin action.

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