



## ORIGINAL ARTICLE

## Effect of dimethoxycurcumin beyond degradation of androgen receptor

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## ABSTRACT

**Background:** Androgen receptor (AR) plays an important role in the pathogenesis of prostate cancer and acne. Dimethoxycurcumin is a newly found enhancer of AR degradation, which highlights its potential for treatment of AR-related disorders. Follicular hyperkeratosis is one essential factor in the complicated pathogenesis of acne, in which some dermatopathologists have observed overexpressed psoriasin, an activator protein (AP)-1-targeted gene product.

**Methods:** We used the HaCaT cell line to determine the effect of dimethoxycurcumin on expression of AP-1 subunits and AP-1-targeted genes, psoriasin and cyclin D1, by luciferase reporter assay, western blotting and reverse transcriptase polymerase chain reaction. We also used flow cytometry to analyze changes in cell populations in response to increasing dose of dimethoxycurcumin.

**Results:** Dimethoxycurcumin inhibits psoriasin promoter activities at the basal or enhanced level induced by exogenous c-Jun/c-Fos heterodimeric AP-1. Expression of endogenous c-Jun and c-Fos, two important subunits of dimeric AP-1, was downregulated at the mRNA or protein level by dimethoxycurcumin in HaCaT cells. Inhibition of endogenous cyclin D1 occurred at both the transcript and protein levels. A shift of subpopulations of cells into sub-G1 phase was consistent with reduced cyclin D1, corresponding to dimethoxycurcumin treatment.

**Conclusion:** Identification of AP-1 transcription factor as a specific target for dimethoxycurcumin-downregulated molecules in human keratinocytes suggests that this novel chemical modulates various AP-1-related events in the epidermis, including cell-cycle progression and its role as an inflammatory reservoir. We provided evidence for this because expression of cyclin D1 and psoriasin, two AP-1-regulated gene products, was inhibited by dimethoxycurcumin in HaCaT and HeLa cells. Cyclin D1 is an important cell-cycle regulator, whereas psoriasin is a potent cytokine for innate immunity. We anticipate that more diseases will benefit from this curcumin analog in the near future.

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## Introduction

Acne vulgaris is an inflammatory skin disorder that affects many people worldwide. Besides the physical discomfort, the cosmetic sequelae can have a major psychosocial impact on the patient's life.<sup>1</sup> In some cases, acne also lowers self-esteem, which may lead to significant depressive mood.<sup>2</sup> A large body of evidence also indicates that acne may impair the patients' daily quality of life, to

which physicians need to pay more attention to achieve a satisfactory therapeutic goal.<sup>3,4</sup>

Pathogenesis is significantly related to androgen production, or more exactly, androgen sensitivity.<sup>5–7</sup> This implies that androgens and their receptors play important roles in acne formation. Acne is known to result from a recalcitrant inflammatory process caused by *Propionibacterium acne* infection in the blocked follicles on the affected skin. The blockage results from follicular hyperkeratosis located at the orifice, which causes the retention of overproduced sebum and leads to *P. acne* overgrowth. The bacterial overgrowth induces innate antibacterial peptides such as psoriasin, which is a strong neutrophil attractant and it also enhances the hyperproliferation of epidermal keratinocytes.<sup>8–12</sup> We can easily tell that there is a vicious cycle for the inflammatory acne to progress.

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A lot of medications have been used to treat acne and these have led to variable outcomes in individual patients.<sup>13</sup> In general, these therapeutic agents do not cover all the above-mentioned aspects, but alone they can interrupt the vicious inflammatory cycle, or in combination, they can cover most aspects of the ongoing inflammation. For example, topical agents that contain benzoyl peroxide at a concentration of 5–10% can inhibit *P. acne* overgrowth within lesions. Benzoyl peroxide also exhibits significant anti-inflammatory and keratolytic activities.<sup>14</sup> Tetracyclines can also inhibit *P. acne* due to its antibiotic property, but it is also known that tetracyclines exhibit an anti-inflammatory effect through some mechanisms that await further exploration.<sup>15–17</sup> Retinoids have been used to treat acne because they can inhibit follicular hyperkeratosis and sebum production, although they exert no significant effect on the antibacterial or anti-inflammatory aspects.<sup>18</sup>

A novel agent, dimethoxycurcumin, a curcumin analog, has potential for treatment of prostate cancer and spinal and bulbar muscular atrophy.<sup>19–21</sup> It is known that part of the reason for recurrence of prostate cancer is the intrinsic escape mechanism developed in the cancer cells.<sup>22,23</sup> For example, prostate cancer cells may acquire a ligand-independent way to waive the necessity of hormones for cell growth. Intracellular overexpressed ARs have been found to play a role in recalcitrant prostate cancer. The newly found dimethoxycurcumin is a highly selective inhibitor that works through a pathway to induce degradation of AR, thereby leading to apoptosis of the prostate cancer cells.<sup>21</sup> It seems promising but awaits further clinical trials. Likewise, it may also be used to treat acne because androgens play an important role in the pathogenesis of acne. Dimethoxycurcumin has been used in an early clinical trial to test its application for acne treatment (available at: <http://clinicaltrials.gov/ct2/show/NCT00525499>).

Additionally, it has been reported previously that androgen deprivation downregulates S100P expression in LNCaP prostate cancer cells.<sup>24</sup> Interestingly, many of the members of the S100 protein family, including S100P and psoriasin (also known as S100A7), can be detectable in normal or diseased epidermis.<sup>10,25</sup> As we mentioned earlier, psoriasin is upregulated in acne lesions.<sup>8,9</sup> AR is a well-known ubiquitous transcription factor whose target genes regulate many cellular functions including survival, proliferation and differentiation, therefore, we established experiments to explore whether dimethoxycurcumin affects the expression of psoriasin, which plays a dual role in inflammation and epidermal hyperplasia. Importantly, our work disclosed that, beyond ARs, activator protein (AP)-1 is another transcription factor that is inhibited by dimethoxycurcumin.

## Methods

### Plasmids

Reporter gene psoriasin (–743/+1)-LUC was constructed by the polymerase chain reaction (PCR) product cut with *KpnI/XhoI* and sub-cloned into the *KpnI* and *XhoI* sites of the pGL3-SV40-LUC vector. Various AP-1 proteins fused with the pCI vector were a gift from D Chalbos (Institute National de la Santé et de la Recherche Médicale, France), served as PCR templates for various AP-1-coding regions and were then sub-cloned into the *EcoRI* and *XhoI* sites of the pSG5.HA vector, which has promoters for *in vitro* and mammalian cell expression with an N-terminal HA-tag.<sup>26</sup>

### Cell culture, reagents and transient transfection assays

For functional assays, HeLa and HaCaT cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%

charcoal/dextran-treated fetal bovine serum (Life Technologies, Carlsbad, CA, USA). Transient transfection (jetPEI, PolyPlus-transfection) luciferase assays (Promega, Madison, WI, USA) were performed in 24-well culture dishes as described previously.<sup>27,28</sup> Total DNA was adjusted to 1 µg by adding the necessary amount of the respective empty vector. The transfected cell extract luciferase activity is presented in relative light units and expressed as the mean and standard deviation from three transfected cultures. Dimethoxycurcumin was purchased from the Cayman Chemical Company, Ann Arbor, MI, USA.

### Cell viability assay

Cells were seeded in 96-well culture plates and were allowed to grow for 1 day. The cells were then exposed to the indicated concentration of dimethoxycurcumin in fresh DMEM for 24 hours. 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL in phosphate-buffered saline; PBS) was added to each well and the plate was incubated for 2 hours at 37 °C. Dimethylsulfoxide (DMSO) (E.Merck, Darmstadt, West Germany, 150 µl) was then added as a solubilizing agent and the absorbance at 540 nm was measured using an ELISA plate reader (Multiskan EX, Thermo, USA). As a control, cells treated with medium containing no dimethoxycurcumin were set as 100% cell survival.

### Immunoblotting

The cell extracts were lysed in RIPA buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, and 1% Triton 100) at 4 °C, separated by SDS-PAGE and analyzed via immunoblotting, with antibodies against c-Fos, cyclin D1 and actinin (ACTN) (Santa Cruz, Biotechnology, Santa Cruz, CA, USA).

### Reverse transcriptase PCR

Total RNA was extracted from growing cells using a total RNA reagent kit (Biomax, Taiwan) according to the manufacturer's instructions. One microgram of total RNA was subjected to reverse transcription using Moloney Murine Leukemia Virus reverse transcriptase for 1 hour at 37 °C (Epicentre Biotechnologies, Madison, WI, USA). PCR was performed in the linear range (30 cycles) with primers specific for *cyclin D1*, *c-Fos*, *c-Jun* and *GAPDH*. The sequences of the primers for the amplification of target genes were as follows: *cyclin D1* top-strand, 5'-ATGGAACACCAGCTCCTGTGCTGC-3'; *cyclin D1* bottom-strand, 5'-TCAGATGTCCACGTCGCCGACGTCGG-3'; *c-Fos* top-strand, 5'-GACTACGAGGCGTCATCCTC-3'; *c-Fos* bottom-strand, 5'-GCTCTGGTCTGCGATGGGGCC-3'; *c-Jun* top-strand, 5'-ATGACTG CAAAGATGGAAACGACC-3'; *c-Jun* bottom-strand, 5'-TCGGCGAA GCCCTCCTGCTC-3'; *GAPDH* top-strand, 5'-AACGGATTGGCCGTAT TGGG-3'; *GAPDH* bottom-strand, 5'-GGGATGACCTTGCCACAC CC-3'. The thermocycling conditions were as follows: one cycle at 95 °C for 5 minutes followed by 30 cycles of 95 °C for 45 seconds, 55 °C for 30 seconds, and 72 °C for 40 seconds. Amplified products were subjected to 1.2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

### Fluorescence-activated cell sorting

This analysis was based on the measurement of DNA content of nuclei labeled with propidium iodide. For cell cycle evaluation, cells were treated as for the proliferation experiments, washed with ice-cold PBS and incubated with propidium iodide (0.05% mg/mL in PBS, 0.1% Triton X-100, and 0.01% RNase) for 15 minutes

at room temperature in the dark. Cells then were subjected to fluorescence-activated cell sorting, and analysis of the cell cycle was evaluated using FACSCalibur flow cytometry (Becton–Dickinson Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using the Cell Quest Pro software package (Becton–Dickinson Biosciences).

**Statistical analysis**

Comparisons between groups were determined by a two-tailed *t* test (SPSS Advanced Statistics 17.0). A *p* value <0.05 was reported as a significant difference between groups.

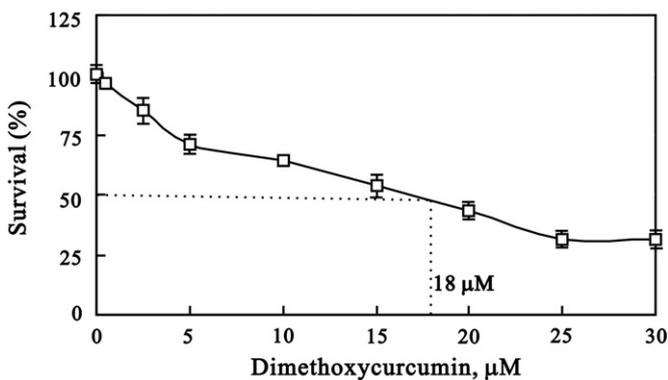
**Results**

**Dimethoxycurcumin decreases viability of HaCaT cells**

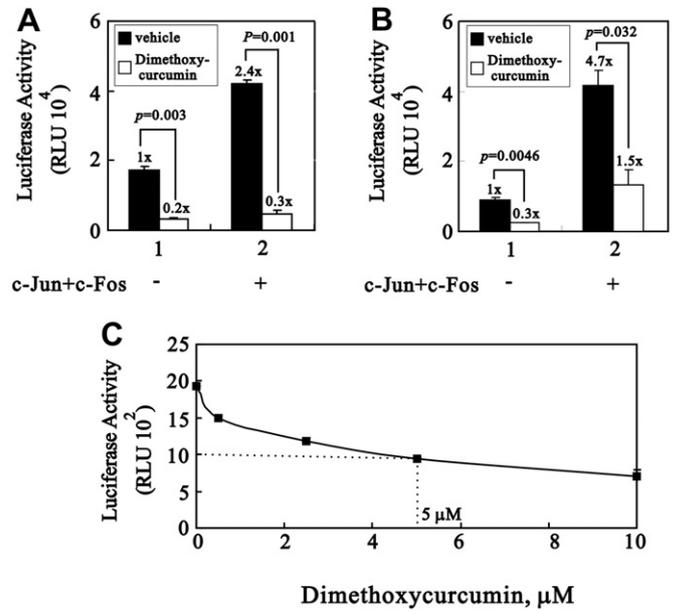
We measured the viability of HaCaT cells in the presence of dimethoxycurcumin using the MTT assay. The IC<sub>50</sub> for HaCaT cells incubated for 24 hours was around 18 μM (Figure 1), which is higher than that against prostate cancer cell lines.<sup>21</sup>

**Dimethoxycurcumin inhibits AP-1 transcriptional activity and psoriasis-promoter activity**

Psoriasis has been shown to be overexpressed in the keratinocytes of acne lesions.<sup>8,9</sup> It has been reported that there is an AP-1 binding site in the psoriasis promoter, therefore, we established experiments to evaluate psoriasis promoter activity using luciferase reporter assay, with or without transfection of exogenous c-Jun and c-Fos, to reconstitute the prototypic heterodimeric AP-1 transcription factor. Overexpressed c-Jun/c-Fos indeed activates the psoriasis promoter (Figure 2A, comparing column 2 to column 1, closed bar; *p* = 0.003). Interestingly, in the presence of dimethoxycurcumin, the AP-1-activated promoter activity was reduced, indicating dimethoxycurcumin negatively regulates the transcriptional activity of AP-1 (Figure 2A, column 2, comparing closed bar to open bar). In support, reduced basal promoter activity indicated that the endogenous transcriptional activators for psoriasis promoter were also inhibited by dimethoxycurcumin (Figure 2A, column 1, comparing closed bar to open bar). This prompted us to start a detailed investigation of the effect of dimethoxycurcumin treatment on the endogenous AP-1 in human keratinocytes.

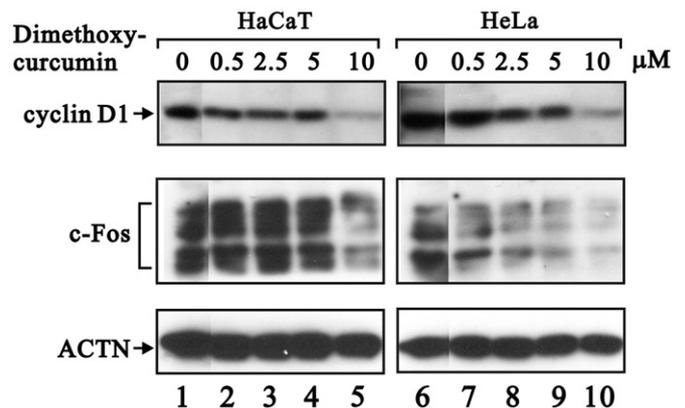


**Figure 1** IC<sub>50</sub> of dimethoxycurcumin in HaCaT cells. HaCaT cells were treated with indicated amount of dimethoxycurcumin for 24 hours. MTT assays were performed at the indicated times. Results are representative of two independent experiments.



**Figure 2** Effects of dimethoxycurcumin on psoriasis promoter activity in HaCaT and HeLa cells. HaCaT (A) and HeLa (B) cells were co-transfected with 0.25 μg psoriasis(-743/+1)-LUC and, where indicated, 0.25 μg empty vector or c-Jun/c-Fos complex. The transfected cells were treated with vehicle (closed) or 5 μM dimethoxycurcumin (open) for 36 hours. (C) HaCaT cells were transfected with 0.25 μg psoriasis(-743/+1)-LUC and treated with indicated amount of dimethoxycurcumin for 36 hours. Luciferase activities in the transfected cell extracts were then determined. The numbers above the columns indicate the luciferase activity relative to an index of 1 for samples to which neither AP-1 nor dimethoxycurcumin was added. These data are the average of three independent experiments (mean ± standard deviation; *n* = 3). RLU = relative light units.

Moreover, similar results were observed using HeLa cells (Figure 2B), suggesting that inhibition of AP-1 by dimethoxycurcumin might be a general instead of a cell-specific event. The concentration of dimethoxycurcumin to reduce 50% of psoriasis promoter activity in HaCaT cells was measured at 5 μM (Figure 2C), which is the concentration that we chose to perform the luciferase activity assays (Figure 2A and 2B).



**Figure 3** Effects of dimethoxycurcumin on protein expression of cyclin D1 and c-Fos in HaCaT and HeLa cells. HaCaT and HeLa cells were treated with indicated amount of dimethoxycurcumin for 24 hours. Cells were subject to immunoblotting for detection of cyclin D1, c-Fos and ACTN (loading control). The data shown here are the representative of three independent experiments.

### Dimethoxycurcumin inhibits protein level of c-Fos and cyclin D1

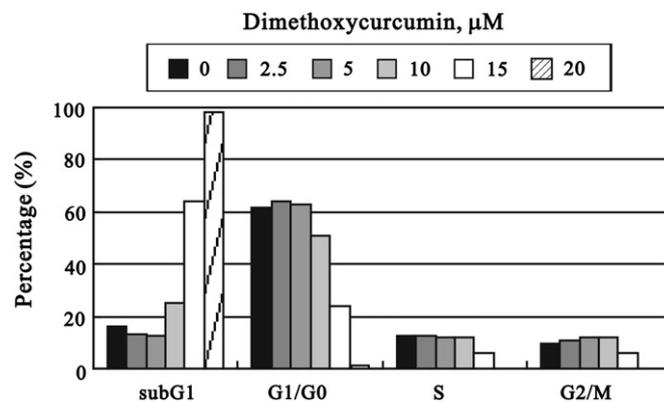
Using western blotting, we checked the amount of endogenous c-Fos protein, one subunit of AP-1, and cyclin D1, which is also an AP-1-targeted gene product.<sup>29</sup> As anticipated, the expression of cyclin D1 was inhibited by dimethoxycurcumin in a dose-dependent manner (Figure 3, lanes 1–5, upper panel). Interestingly, the expression of c-Fos showed a similar pattern, indicating that endogenous AP-1 may be a downregulating target for dimethoxycurcumin (Figure 3, lanes 1–5, middle panel). Again, a similar theme was also observed in HeLa cells (Figure 3, lanes 6–10).

### Dimethoxycurcumin inhibits transcription of c-Jun but not c-Fos genes

Using RT-PCR, we determined whether the inhibition of endogenous AP-1 took place at the transcription level. Figure 4 shows that, in the presence of 15  $\mu$ M dimethoxycurcumin, transcription of c-Jun decreased in a time-dependent manner (lanes 1–6, uppermost panel). However, a similar effect was not observed for c-Fos transcription (upper second panel). In contrast, the transcription of cyclin D1 was apparently inhibited. The downregulated transcription of cyclin D1 gene supports the inhibitory effect of dimethoxycurcumin on the transcriptional activity of AP-1 (Figure 1), although other factors in addition to AP-1 may also be affected by dimethoxycurcumin (see Discussion).

### Sub-G1 population of HaCaT cells increased with treatment with dimethoxycurcumin in a dose-dependent manner

Cyclin D1 promoted G1/S transition in the cell cycle. The amount of cyclin D1 was decreased by dimethoxycurcumin, therefore, we used flow cytometry to observe the distribution of the cell population as a function of cyclin D1 in the presence of increasing concentrations of dimethoxycurcumin. The HaCaT cell population



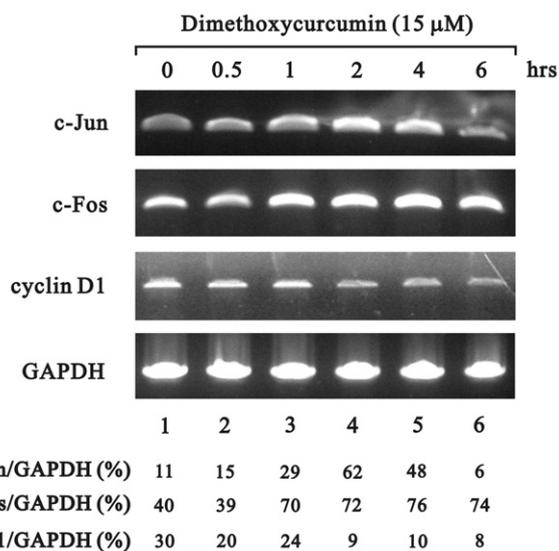
**Figure 5** Cell cycle profiles of HaCaT cells treated with dimethoxycurcumin. HaCaT cells were treated with indicated amount of dimethoxycurcumin for 36 hours. Fixed cells were stained with propidium iodide, and DNA content was determined by fluorescence-activated cell sorting analysis. The results are representatives of three independent experiments.

apparently shifted towards the subG1 phase, particularly as concentration increased towards the IC<sub>50</sub> (Figure 5).

### Discussion

We demonstrated that dimethoxycurcumin inhibited psoriasis promoter activity at both basal and AP-1-activated levels. What is important for us is that psoriasis is one of the innate antimicrobial peptides that are secreted by keratinocytes.<sup>25</sup> The review of Watson et al has shown that psoriasis may attract inflammatory cells to inflammatory skin lesions and cause epidermal hyperplasia.<sup>10</sup> The prevention of keratinocytes from production of psoriasis by treatment with dimethoxycurcumin would be beneficial for acne control because follicular hyperkeratosis and local inflammation would be alleviated. This awaits pathological verification when the topical medication is available for further clinical investigation.

Interestingly, psoriasis promoter has been shown to harbor an AP-1-binding site, which is a positive regulatory *cis*-acting element defined in the keratinocyte cell line KHSV40.<sup>30</sup> We used human keratinocyte cell line HaCaT to demonstrate that the luciferase reporter activity driven by psoriasis promoter can be activated by the AP-1 transcription factor, whose prototype is c-Jun/c-Fos heterodimer, as shown in several previous studies.<sup>31–34</sup> AP-1, similar to AR, is also ubiquitously present in different types of cells, and it is actively engaged in many aspects of skin physiology, including epidermal homeostasis and carcinogenesis.<sup>32,35–39</sup> Moreover, our data indicate that not only AR, but also AP-1, is the target for dimethoxycurcumin to carry out its pharmacologic effects, such as induction of apoptosis (Figure 1 and ref. 21). The amount of AP-1 complex in human keratinocytes may be reduced by dimethoxycurcumin because the expression level of both endogenous c-Jun and c-Fos is repressed. In support, we observed a reduced protein level of cyclin D1, which is an AP-1-responsive gene product (Figure 2). However, the molecular mechanisms are not the same for the regulation of c-Jun and c-Fos expression. Inhibition of c-Jun expression level occurs at the transcriptional level because we observed that the amount of mRNA transcript was reduced, as shown by RT-PCR. For c-Fos, the amount of protein but not transcript was decreased after dimethoxycurcumin treatment. We speculate that the inhibition of c-Fos expression by dimethoxycurcumin may occur at the post-transcriptional or even post-translational level. It would be interesting to investigate further



**Figure 4** Effects of dimethoxycurcumin on transcription of c-Jun, c-Fos and cyclin D1 in HaCaT cells. HaCaT cells were treated with 15  $\mu$ M dimethoxycurcumin for indicated time where mRNA was collected and then assessed by RT-PCR. The data shown here are representative of three independent experiments. The intensity of each band was quantitated and expressed as percentage relative to the intensity of respective glyceraldehyde 3 phosphate dehydrogenase transcript (internal control) collected at the same indicated timepoints. RT-PCR = reverse transcriptase polymerase chain reaction.

what makes the difference and whether c-Fos degradation is also enhanced by dimethoxycurcumin; similar to the lessons learned from AR.<sup>21,40</sup>

As we know, cyclin D1 is one of the important regulators for the transition from G1 to S phase in the cell cycle.<sup>41,42</sup> We observed reduced cyclin D1 expression at both the transcript and protein levels. The inhibition of cyclin D1 expression by dimethoxycurcumin indicates that this novel agent might inhibit the proliferation of human keratinocytes through interference with the cell cycle. Moreover, the dimethoxycurcumin-induced reduction in cyclin D1 and c-Fos has also been observed in HeLa cells, in which sex hormones and AP-1 also affect gene expression significantly.<sup>43,44</sup> Our data imply that dimethoxycurcumin may also have a potential role in interfering with the pathogenesis of cervical cancer. In addition, the protein level of cyclin D1 began to decrease at lower concentrations of dimethoxycurcumin compared with that for c-Fos, as shown by western blotting in HaCaT cells (Figure 3). This might have resulted from inhibition of some other activators such as ternary complex factor or nuclear factor- $\kappa$ B, whose binding sites are highly conserved across species and crucial for regulation of cyclin D1 expression. These unexplored factors might also be the potential targets for dimethoxycurcumin.<sup>45</sup> Moreover, formation of the derepression complex containing c-Jun/c-Fos/estrogen receptor, which is able to displace the repressor complex containing Ying-Yang 1 and histone deacetylase 1 might also be disrupted because the amount of Jun/Fos subunits may be decreased by dimethoxycurcumin treatment.<sup>46</sup>

In summary, dimethoxycurcumin might inhibit the follicular hyperkeratosis shown in acne lesions by downregulating two factors, psoriasis and cyclin D1. Dimethoxycurcumin might be of benefit for acne control, and interfere with carcinogenesis of various cell types. Moreover, our data expand the repertoire of transcriptomes that are affected by dimethoxycurcumin, including those related to AR and AP-1, and other transcription factors await exploration.

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