

Effects of demethoxycurcumin on the viability and apoptosis of skin cancer cells

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Abstract. The present study investigated the effects and mechanisms of demethoxycurcumin (DMC) on a human skin squamous cell carcinoma cell line, A431, and a human keratinocyte cell line, HaCaT. A431 and HaCaT cells were cultured *in vitro*. The effects of DMC treatment on cell viability were analyzed using the Cell Counting kit-8 (CCK-8) assay; cell cycle distribution was analyzed by flow cytometry; apoptosis was assessed by flow cytometry and Hoechst 33258 staining; and the protein expression levels of cytochrome *c*, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (BAX), caspase-9 and caspase-3 were evaluated by western blotting. CCK-8 assay results demonstrated that DMC treatment significantly inhibited viability of A431 and HaCaT cells in a dose-dependent manner. Flow cytometric analysis confirmed that DMC treatment induced apoptosis in a dose-dependent manner, and significantly increased the proportion of cells in G₂/M phase. Western blot analysis indicated that the protein expression levels of Bcl-2 were decreased, whereas the expression levels of BAX, caspase-9, caspase-3 and cytochrome *c* were increased following DMC treatment compared with in untreated cells. In conclusion, DMC treatment significantly inhibited viability of A431 and HaCaT cells, and induced cell cycle arrest in G₂/M phase. The present study indicated that

DMC may induce apoptosis of skin cancer cells through a caspase-dependent pathway.

Introduction

Squamous cell carcinoma (SCC) is derived from malignant transformation of epithelial cells, including cells of the epidermis. SCC is a common skin malignancy worldwide (1), and its occurrence has significantly increased in recent years. SCC is highly aggressive, grows quickly and has an elevated frequency of metastasis; Squamous cell carcinoma of the skin is frequently treated by surgical excision, Mohs surgery or electrodesiccation and curettage (2,3). The disadvantages of these treatments are trauma and scar formation, and are only applied to small skin lesions. Radiation therapy and traditional chemotherapy is a primary treatment option for patients where surgery is not feasible, and is an adjuvant therapy for those with metastatic or high-risk cutaneous SCC (4). However, radiation is not suitable for the distal part of the limbs, areas that difficult to heal and the elderly (5), and traditional chemotherapy has limited application due to the side effects that it causes, including erosion, ulcer and erythema. Cryotherapy and laser treatment are additionally used as for SCC in the clinical setting; however, they have largely been replaced by surgery due to the high recurrence rate (6,7). Photodynamic therapy has received more attention as an effective treatment for SCC of the skin (8), although the curative effect is difficult to compare with other treatments. Therefore, novel treatments targeting SCC are of utmost interest for patients and clinicians (9).

Curcumin (CUR), which is a phenolic pigment extracted from the *Curcuma longa* rhizome, is the main active ingredient of turmeric, and has anti-inflammatory, antioxidant and anticancer properties (10-13). However, CUR is easily degraded *in vitro* and *in vivo*. Demethoxycurcumin (DMC) is a derivative of CUR lacking a methoxy group attached to a benzene ring (Fig. 1); it has similar biological properties to CUR, but is more chemically stable (14).

Previous studies have demonstrated that DMC strongly inhibits proliferation in numerous types of cancer, including prostate cancer, kidney cancer and breast cancer cells (15-17). However, the effects of DMC on skin cancer cells remain

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unknown. In the present study, the effects of DMC treatment on the viability and apoptosis of human skin squamous carcinoma A431 cells and human keratinocyte HaCaT cells were examined. In addition, the molecular mechanisms underlying these effects were explored. The present findings provide a theoretical basis for the potential clinical application of DMC in the treatment of skin cancer.

Materials and methods

Cell culture and reagents. A431 and HaCaT cells were purchased from the Shanghai Cell Collection (Shanghai, China) and the Fuxiang Institute of Biotechnology (Shanghai, China), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin, in a humidified incubator at 37°C supplied with 5% CO₂. Once cells reached 80% confluence, they were passaged at a 1:3 ratio by dissociation with 0.25% trypsin and 0.02% EDTA. Cells in the logarithmic growth phase were used in all experiments. DMC (>99% purity) was obtained from the YuanYe Institute of Biotechnology (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) to generate a 100 mmol/l stock solution. The stock solution was stored at -20°C and freshly diluted in complete culture medium prior to use. The final concentration of DMSO applied to the cells was <0.1%. Rabbit polyclonal primary antibodies against B-cell lymphoma 2 (Bcl-2; cat. no. AB40415), Bcl-2-associated X protein (BAX; cat. no. AB40636), caspase-3 (cat. no. AB42437), caspase-9 (cat. no. AB32539) and cytochrome c (cat. no. AB133504) were purchased from Biogot Technology Co., Ltd. (Nanjing, China). GAPDH (cat. no. sc-32233) was used as a loading control (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The goat-anti-rabbit secondary antibody (cat. no. sc-362262) was purchased from Santa Cruz Biotechnology, Inc.

Cell viability assay. The effects of DMC on A431 and HaCaT cell viability were determined using a Cell Counting kit-8 (CCK-8) assay (Vicmed Co., Ltd., Xuzhou, China). Cells were seeded in 96-well plates at a density of 3x10³ cells/well and incubated overnight in DMEM containing 10% FBS. Cells were treated with various concentrations of DMC (0, 5, 10, 20, 40 and 80 μM) for 24, 48, 72 and 96 h, and were then incubated with CCK-8 reagent for 2 h at room temperature. Absorbance at 450 nm was measured using a microplate spectrophotometer (Thermo Labsystems, Santa Rosa, CA, USA). Inhibition of viability was determined relative to the control group measurement (untreated cells). The IC₅₀ value was calculated using Graphpad Prism software (Graphpad Software, Inc., San Diego, CA, USA). All experiments were performed in triplicate.

Cell cycle distribution assay. Cells were seeded at 1x10⁶ cells/well in 6-well plates, and allowed to grow for 24 h. Cells were treated in triplicate with 0, 5, 10, 20, 40 and 80 μM DMC for 48 h. Following dissociation with 0.25% trypsin, cells were centrifuged at 2,000 rpm for 5 min at room temperature. The supernatant was discarded, and pellets were washed once

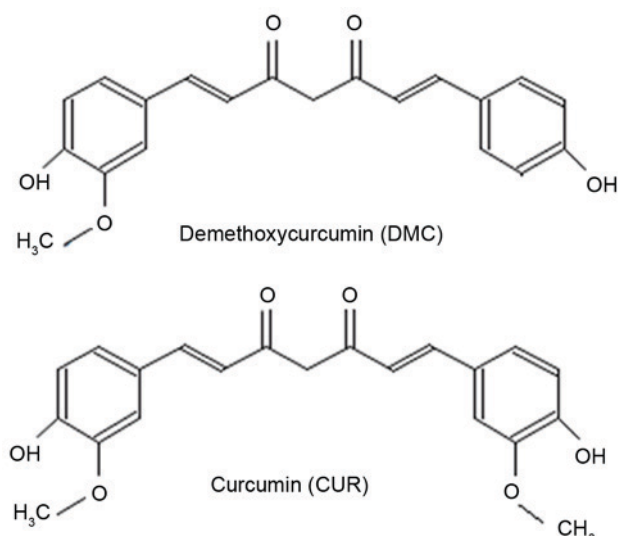


Figure 1. Chemical structure of CUR and DMC. Curcuminoids are phenolic coloring compounds that can be extracted from the rhizomes of *Curcuma longa*. DMC, an analogue of CUR, is one such compound. Compared to CUR, DMC lacks one methoxy group directly linked to a benzene ring. CUR, curcumin; DMC, demethoxycurcumin.

with PBS. Cell pellets were resuspended in 500 μl 70% cold ethanol and fixed overnight at 4°C. Following fixation, pellets were washed with PBS, 100 μl RNase A solution was added, and samples were incubated in a 37°C water bath for 30 min. Subsequently, 400 μl propidium iodide (PI) (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was added, and samples were incubated at 4°C in the dark for 30 min. Samples were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using FCS-express version 3 software (De Novo software, Glendale, CA, USA). The experiment was performed in three independent repeats.

Quantification of apoptosis by flow cytometry. A431 and HaCaT cells were cultured in 6-well plates for 12 h and then treated with 0, 5, 10, 20, 40 and 80 μM DMC for 48 h. Cells were dissociated, collected by centrifugation at 200.67 x g for 5 min at room temperature, and washed twice with PBS. Cells (~1x10⁶) were then resuspended in 500 μl binding buffer containing 5 μl Annexin V and 5 μl PI reagents (Nanjing KeyGen Biotech Co., Ltd.). Following a 15 min incubation at room temperature in the dark, the samples were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using the FCS-express V3 software (de novo software, Thornhill, Ontario, Canada).

Hoechst 33258 DNA staining. A431 and HaCaT cells were seeded in 6-well plates and treated with 10, 20, 40 and 80 μM DMC for 48 h. Untreated cells served as control. Following treatment, the medium was discarded, the cells were washed twice with PBS, incubated with Hoechst 33258 (Nanjing KeyGen Biotech Co., Ltd.) for 5-10 min at room temperature in the dark, washed twice with PBS, and finally observed under a fluorescence microscope.

Western blotting. Adherent and floating cells were harvested at 200.67 x g for 5 min at room temperature and washed

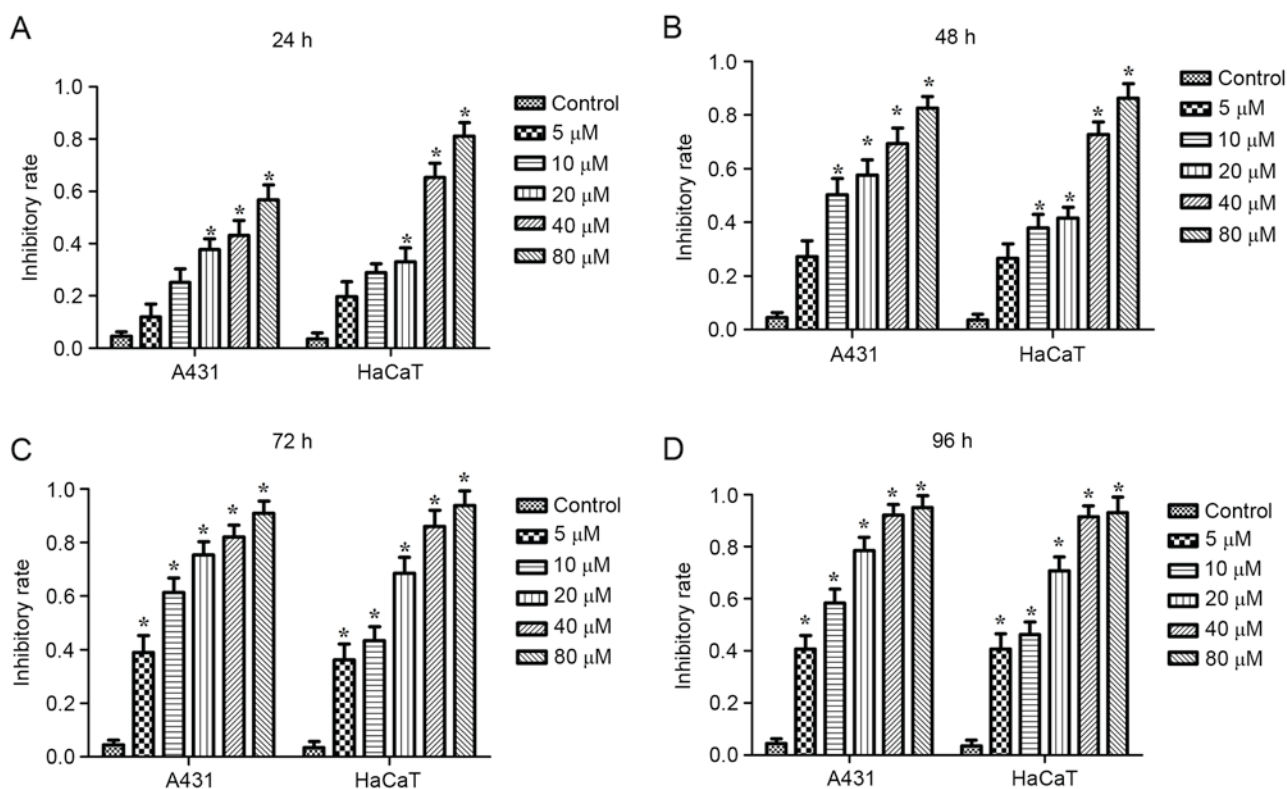


Figure 2. Effects of DMC on the viability of A431 and HaCaT cells. Cells were treated with 5, 10, 20, 40 or 80 μM DMC for (A) 24, (B) 48, (C) 72 and (D) 96 h, and inhibition of cell viability was determined by Cell Counting kit-8 assay. Results are expressed as the mean \pm standard deviation of three independent experiments (n=3). *P<0.05 compared with untreated cells. DMC, demethoxycurcumin.

twice with ice-cold PBS. Cells were homogenized in radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 100 mM PMSF. Homogenates were centrifuged at 15,000 \times g at 4°C for 20 min and the supernatants were collected. Protein concentrations were measured using the bicinchoninic acid method (Nanjing KeyGen Biotech Co., Ltd.), using bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to generate the standard curve. A total of 100 μg protein of each sample was loaded in SDS sample buffer and heat denatured at 100°C for 5 min. Protein samples were then electrophoretically separated by 10-15% SDS-PAGE and transferred onto nitrocellulose membranes (Promega Corporation, Madison, WI, USA). Blocking was performed for 1 h at room temperature in blocking buffer containing 5% non-fat dry milk. Membranes were then incubated with primary antibodies at 4°C overnight (1:1,000 dilution), followed by horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h (1:5,000 dilution). Signals were detected with SuperSignal enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). All experiments were performed in triplicate. Densitometric analysis was performed on protein bands using ImageQuant™ TL software version 8.1; Molecular Dynamics; GE Healthcare Life Sciences, Chalfont, UK) (18).

Statistical analysis. Data were expressed as the mean \pm standard deviation. Statistical significance was determined by independent samples t-test and one-way analysis of variance

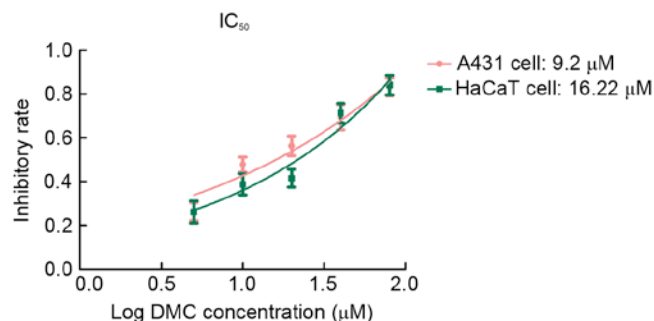


Figure 3. IC₅₀ values for DMC. The IC₅₀ values for DMC were determined after 48 h of DMC treatment in A431 and HaCaT cells using Graph Pad 5.0 software. IC₅₀, half maximal inhibitory concentration; DMC, demethoxycurcumin.

followed by the Tukey's post-hoc test, as appropriate, using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference. Statistical graphs were drawn using GraphPad prism (Graph Pad Software, Inc.).

Results

DMC inhibits viability in A431 and HaCaT cells. In order to investigate the effect of DMC treatment on A431 and HaCaT cell viability, A431 and HaCaT cells were exposed to various concentrations of DMC for 24, 48, 72 or 96 h, and then analyzed using a CCK-8 assay. As demonstrated in Fig. 2,

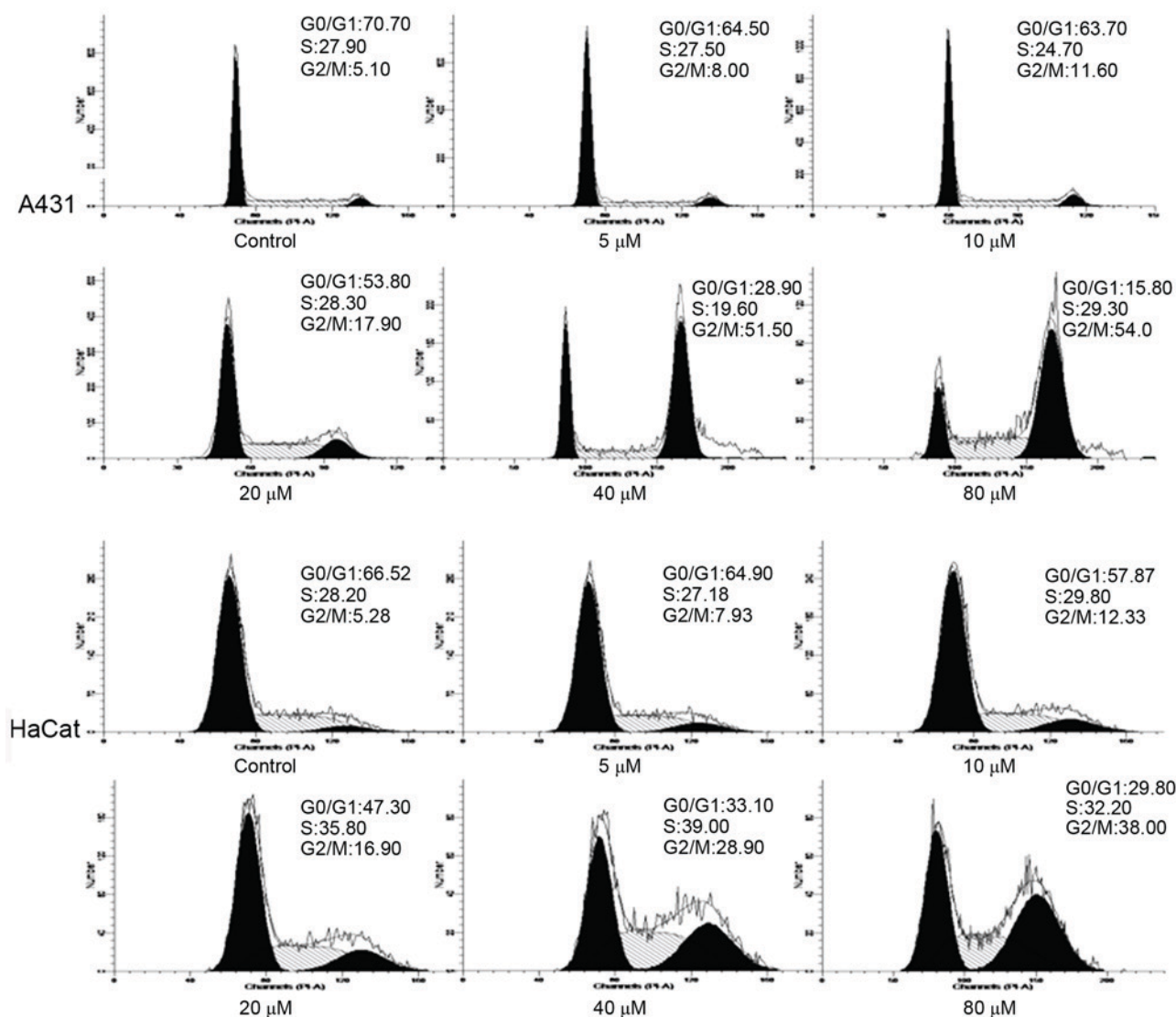


Figure 4. Effects of DMC on cell cycle phase distribution. A431 and HaCaT cells were treated with 5, 10, 20, 40 or 80 μ M DMC for 48 h and cell cycle distribution was analyzed by flow cytometry. Untreated cells served as controls. Representative plots are presented from three independent experiments, with percentage of cells from total cell cycle phase listed in each plot. DMC, demethoxycurcumin.

the cell viability inhibitory rate in DMC-treated cells was significantly increased compared with control cells ($P < 0.05$). The half maximal inhibitory concentration values of DMC for A431 and HaCaT cells at 48 h were 9.2 and 16.22 μ M, respectively (Fig. 3). These results indicated that DMC treatment decreased viability of A431 and HaCaT cells in a dose-dependent manner.

DMC induces G_2/M phase cell cycle arrest in A431 and HaCaT cells. To further analyze the mechanism by which DMC inhibits cell viability, A431 and HaCaT cells were treated with 5, 10, 20, 40 and 80 μ M DMC for 48 h and then analyzed for cell cycle phase distribution by flow cytometry. The results demonstrated that the percentage of cells in the G_0/G_1 phase was markedly decreased, whereas the percentage of cells in the G_2/M phase was markedly increased in the DMC-treated cells compared with untreated cells (Fig. 4). These results indicated that cell cycle arrest at G_2/M may contribute to the inhibitory effects of DMC on cell viability.

DMC induces apoptosis of A431 and HaCaT cells. A431 and HaCaT cells were treated with 5, 10, 20, 40 and 80 μ M DMC for 48 h and apoptosis was measured by Annexin V/PI double staining and flow cytometric analysis. The apoptotic rate of DMC-treated cells was significantly increased in a dose-dependent manner compared with untreated cells ($P < 0.05$; Fig. 5).

Morphological features of DMC-induced apoptosis. To confirm apoptosis by cell morphological observation, A431 and HaCaT cells were exposed to 0, 10, 20, 40 and 80 μ M DMC for 48 h, stained with Hoechst 33258, and observed by fluorescence microscopy. Control untreated cells displayed nuclei with uniform staining (Fig. 6). Conversely, shrinkage of the nuclei was observed in the cells exposed to 20–80 μ M DMC for 48 h (Fig. 6). The number of visibly apoptotic nuclei increased with increasing concentrations of DMC, and various morphological features of apoptosis were observed, including reduction of cell numbers, nuclei with intensely bright staining and fragmented nuclei (Fig. 6).

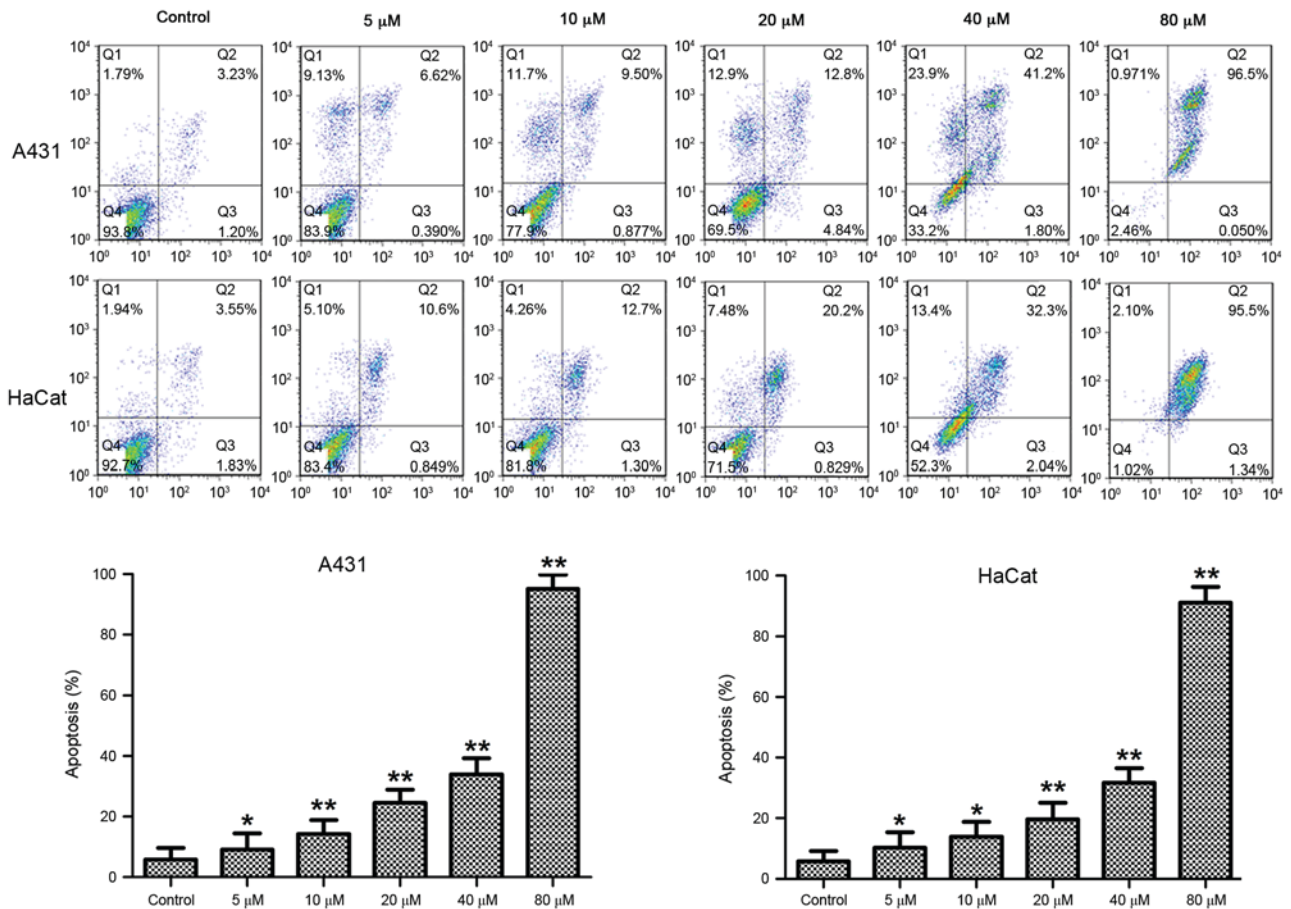


Figure 5. Effects of DMC on apoptosis of A431 and HaCaT cells. Cells were treated with 5, 10, 20, 40 or 80 μM DMC for 48 h, stained with Annexin V and PI, and analyzed by flow cytometry. Untreated cells served as a control. Representative plots are presented from at least three independent experiments. Quantification is presented as mean percentage of apoptotic cells \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ compared with control. DMC, demethoxycurcumin; PI, propidium iodide; Q1, dead cells; Q2, late apoptotic and necrotic cells; Q3, early apoptotic cells; Q4, viable cells.

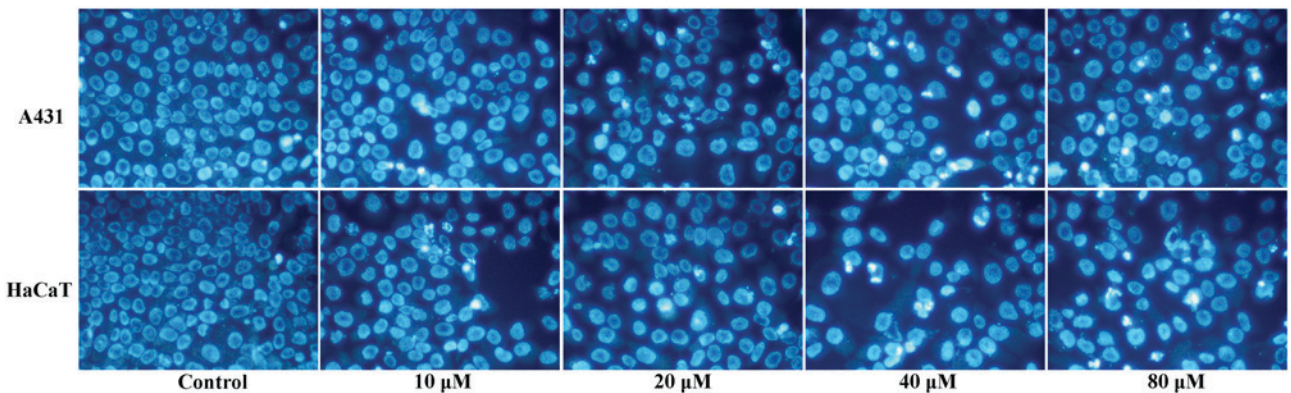


Figure 6. Effects of DMC on apoptotic morphological features. A431 and HaCaT cells were treated with 10, 20, 40 or 80 μM DMC for 48 h, and morphological changes of the nuclei (such as nuclei condensation and fragmentation) were examined by Hoechst 33258 staining and fluorescence imaging. Representative images are presented at original magnification $\times 400$ ($n=3$). DMC, demethoxycurcumin.

DMC increases expression of *Bcl-2*, *BAX*, *caspase-3*, *caspase-9* and *cytochrome c* proteins. In order to explore the mechanisms by which DMC treatment may induce apoptosis, the effects of DMC on the protein expression levels of various established apoptotic markers were examined. A431 and HaCaT cells were treated with 10, 20, 40 and 80 μM DMC for 48 h and then analyzed by western blotting for protein

expression of *Bcl-2*, *BAX*, *caspase-3*, *caspase-9* and *cytochrome c* expression (Figs. 7 and 8). As demonstrated in Fig. 7, DMC treatment resulted in a decrease in the protein expression levels of *Bcl-2*, and an increase in *BAX* expression (Fig. 7). In addition, DMC treatment caused an increase in the protein expression levels of *caspase-9*, *caspase-3* and *cytochrome c* in a dose-dependent manner (Fig. 8).

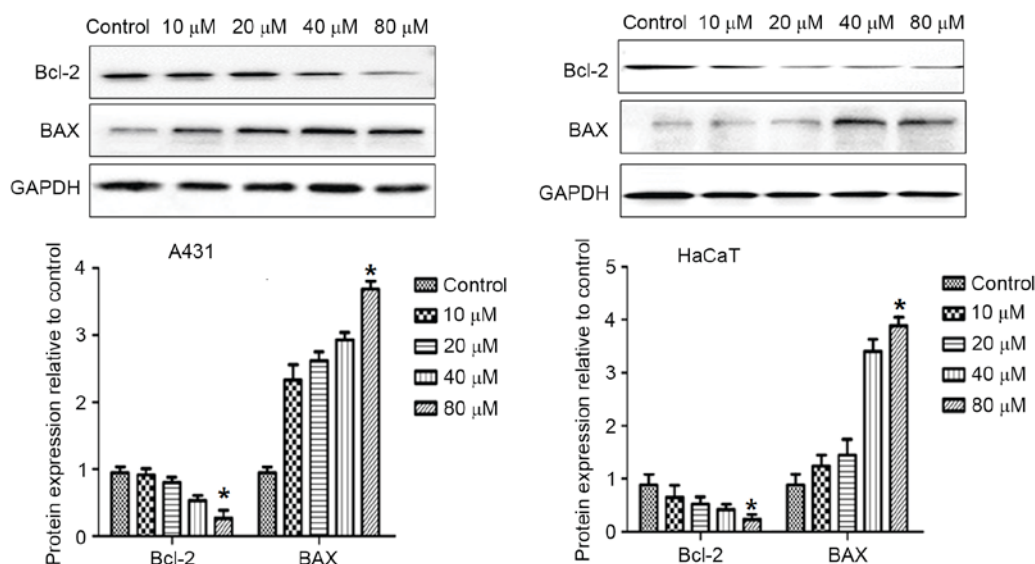


Figure 7. DMC treatment regulates Bcl-2 and BAX protein expression in A431 and HaCaT cells. Cells were treated with 10, 20, 40 or 80 μ M DMC for 48 h and then analyzed by western blotting for protein expression levels of Bcl-2 and Bax. GAPDH was used as a loading control. The bands on the membrane were scanned and their density was analyzed with an Image-J analyzer (LabWorks Software; UVP, Upland, CA, USA). * P <0.05 vs. 40, 20 or 10 μ M DMC. DMC, demethoxycurcumin; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein.

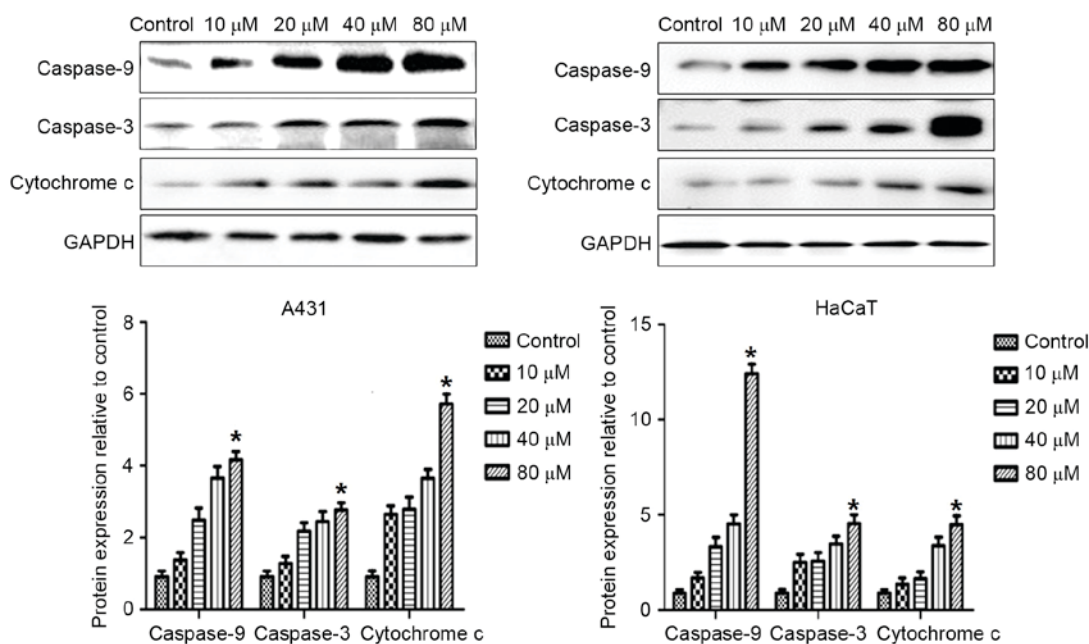


Figure 8. DMC treatment regulates caspase-9, caspase-3, and cytochrome c protein expression in A431 and HaCaT cells. Cells were treated with 10, 20, 40 or 80 μ M DMC for 48 h and then analyzed by western blotting for protein expression levels of caspase-9, caspase-3 and cytochrome c. GAPDH was used as a loading control. Protein bands were scanned and the intensity was determined. * P <0.05 vs. 40, 20 or 10 μ M DMC.

Discussion

Previous studies have demonstrated that CUR exerts inhibitory effects on various types of cancer, including bladder cancer, prostate cancer, colon cancer, liver cancer and breast cancer (19-22). DMC, which is a structural analogue of CUR, also exhibits anti-tumor effects (14) and strongly inhibits proliferation of prostate cancer cells (23). The improved stability of DMC compared to CUR may significantly prolong the time of action of DMC and extend its half-life, suggesting that DMC may be an attractive compound to explore as an anticancer agent.

It has previously been reported that CUR induces cell cycle arrest in G₂/M phase in liver cancer J5 cells (24). In addition, DMC has been demonstrated to induce G₂/M cell cycle arrest in human glioma U87 cells (25). In the present study, the *in vitro* viability of A431 and HaCaT cells treated with various concentrations of DMC was significantly inhibited in a dose-dependent manner. Treatment with DMC reduced the percentage of A431 and HaCaT cells in G₀/G₁ phase in a dose-dependent manner, whereas it increased the percentage of cells in S and G₂/M phases compared with untreated cells, indicating an inhibition of mitosis. Evaluation of apoptosis by various methods,

including Annexin V/PI staining, Hoechst 33258 staining and morphological observation, demonstrated that DMC treatment increased apoptosis of A431 and HaCaT cells, which was consistent with the DMC-mediated inhibition of cell viability.

The stimuli and pathways leading to cellular apoptosis are diverse and complex, and regulation of apoptosis involves proapoptotic and anti-apoptotic genes. The Bcl-2 family proteins serve vital roles in the regulation of apoptosis (26-28). The Bcl-2 family can be divided into two categories: pro- and anti-apoptotic genes. These genes are key factors that determine if a cell will commit to apoptosis or survival, and this decision is determined by the ratio of BAX to Bcl-2 (26,29). Bcl-2 family members alter mitochondrial membrane permeability and regulate the release of cytochrome *c* from the mitochondria to the cytoplasm. Cytochrome *c* then regulates cell apoptosis through the transmission and amplification of apoptotic signals in the cytoplasm. The close relationship between cytochrome *c* and caspase family members has been detailed (30). Caspases are a specific kind of protease (31). At present, numerous caspases have been identified: Caspases-8, 9 and 10 initiate cellular apoptosis, whereas caspases-3, 6 and 7 are involved in implementation of the apoptotic process. Cytochrome *c* interacts with apoptotic peptidase activating factor 1 to form an apoptotic complex (32,33). Caspase-9 is recruited and activated by this apoptotic complex, which then activates caspase-3. Caspase-3 is one of the most important apoptotic execution factors in the caspase family, and its activation is a sign of the irreversible commitment to apoptosis (34). The activation of caspases can in turn further promote the release of cytochrome *c* from the mitochondria, resulting in an overall amplification of the caspase cascade to promote apoptosis.

In the present study, treatment with DMC resulted in a significant dose-dependent reduction in the protein expression levels of Bcl-2, accompanied by a significant increase in BAX, caspase-9, caspase-3 and cytochrome *c*. These results indicated that DMC may regulate the release of cytochrome *c* by downregulating Bcl-2 and upregulating BAX. The release of cytochrome *c* can then activate downstream caspase-9 and caspase-3, leading to an amplification of the caspase cascade reaction, and ultimately to enhanced cell apoptosis.

In conclusion, treatment with DMC in the range of 10-80 μ M inhibited the viability of A431 and HaCaT cells in a dose-dependent manner. This was accompanied by cell cycle arrest in G₂/M phase and induction of apoptosis. These results provide a putative mechanism of action for DMC regarding its potential application as a therapeutic agent for the treatment of skin malignancies.

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