Dihydromyricetin induces cell cycle arrest and apoptosis in melanoma SK-MEL-28 cells

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Abstract. Dihydromyricetin (DHM) exhibits multiple pharmacological activities; however, the role of DHM in antimelanoma activities and the underlying molecular mechanisms are unclear. The aim of the present study was to evaluate the effects of DHM on cell proliferation, cell cycle distribution and apoptosis in the human melanoma SK-MEL-28 cell line, and to explore the related mechanisms. The effect of DHM on cell proliferation was investigated by MTT assay, and cell cycle distribution was determined by flow cytometry. TUNEL assay was used to evaluate DHM-mediated apoptosis, and western blotting was applied to examine expression levels of p53, p21, Cdc25A, Cdc2, P-Cdc2, Bax, IKK-α, NF-κB p65, p38 and P-p38 proteins. The results revealed that DHM suppressed cell proliferation of SK-MEL-28 cells in a concentration- and time-dependent manner, and caused cell cycle arrest at the G1/S phase. DHM increased the production of p53 and p21 proteins and downregulated the production of Cdc25A, Cdc2 and P-Cdc2 proteins, which induced cell cycle arrest. Additionally, DHM significantly induced the apoptosis of SK-MEL-28 cells, and enhanced the expression levels of Bax proteins and decreased the protein levels of IKK-α, NF-κB (p65) and P-p38. The results suggest that DHM may be a novel and effective candidate agent to inhibit the growth of melanoma.

Introduction

The number of new cases of melanoma was estimated at 76,250, and 9,180 patients died of melanoma of the skin in the United States in 2012 (1). Despite a relatively low incidence of melanoma accounting for only 4% of all dermatological cancers, metastatic melanoma is responsible for ~80% of deaths caused by skin cancer due to its aggressive properties and drug-resistance (2,3). When patients are diagnosed with primary melanoma early, surgical resection is the best option for the majority in order to reduce the risk of mortality. However, a recent report revealed that the 1-year overall survival rate in phase II trials for patients with metastatic melanoma was 25%, with a median survival time of 6.2 months (4). Therefore, the search for effective agents to overcome this devastating disease is still the top priority in melanoma therapy.

Dihydromyricetin (DHM) is a flavonoid compound isolated from the classical Chinese herb *Ampelopsis gredersedenta* widely distributed in South China (5,7). DHM is also called ampelopsin (2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one, and its chemical structure is similar to myrecetin (Fig. 1A). DHM was previously reported to exert multiple bioactivities including hepatic protection (8,9), antioxidation (10,11), hypoglycemic activity (12) and antiinflammation (13).

In recent years, researchers have had great interest in exploring the anticancer effects of DHM. A previous study confirmed that DHM inhibits the cell proliferation and metastasis of prostate cancer in vitro and in vivo (14). DHM sodium was shown to inhibit the proliferation of bladder carcinoma, and its molecular mechanism was partially attributed to cell cycle arrest (15). DHM was found to suppress the growth of transplanted tumors derived from human lung cancer GLC-82 cells in nude mice (16). In addition, DHM suppressed the proliferation of hepatoma cells by inhibiting angiogenesis via downregulation of vascular endothelial growth factor and basic fibroblast growth factor expression (17,18).

Additionally, from the PubMed Database, we found that only several articles in regard to the effects of DHM on melanoma have been reported in the past two decades, and those reports only showed DHM-inhibited migration, invasion and adhesion of mouse melanoma B16 cells in vitro, and decreased pulmonary metastasis of B16 cells in mice (19-21). However, the molecular mechanisms remain unclear. Therefore, in light...
of the previous studies, we speculated that DHM may be an effective therapeutic candidate for melanoma therapy. In the present study, we investigated the inhibitory effects of DHM in human melanoma SK-MEL-28 cells and the underlying cellular and molecular mechanisms. We demonstrated that DHM inhibited the proliferation of SK-MEL-28 cells in a concentration- and time-dependent manner and induced cell cycle arrest and promoted the apoptosis of SK-MEL-28 cells.

**Materials and methods**

**Cell line and chemicals.** The human melanoma SK-MEL-28 cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences, and was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified incubator at 37°C with 5% CO₂. Cells were passaged by trypsinization with 0.25% trypsin-EDTA solution (Gibco) for 1-2 min after having grown to 80-90% confluence. Dihydrouridylic acid was purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) for the stock solution. It was then diluted to a final concentration in culture medium for the following studies, and the final concentration of DMSO was <0.3%. TUNEL assay kit for detecting apoptosis was obtained from Promega Corporation (Madison, WI, USA). All primary antibodies used for p53, p21, Cdc25A, Cdc2, P-Cdc2, Bax, IKK-α, NF-xB p65, p38 and P-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Cell cycle staining solution was obtained from Multisciences Biotechnology, Co., Ltd. (Hangzhou, China) (cat# CCS012A).

**Growth inhibitory assay.** The effect of DHM treatment on the inhibition of SK-MEL-28 cells was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Biotechnology) assay according to a previously published study with minor modifications (22). Briefly, SK-MEL-28 cells were cultured in 96-well plates at 1x10⁴ cells/well in 1640 culture medium with 10% FBS. DHM was added after 10 h starting from inoculation of SK-MEL-28 cells. After incubation for 24, 48 and 72 h, 20 µl of MTT solution (5 mg/ml) in 1X phosphate-buffered saline (PBS) was directly added into each well and incubation was continued for 4 h at 37°C. Thereafter, the medium was removed, and 150 µl of DMSO was added to the wells. The absorbance was measured at a wavelength of 570 nm with an automated spectrophotometric plate reader (Perkin-Elmer, Waltham, MA, USA). The experiments were independently performed at least three times.

**DHM regulates cell cycle progression in SK-MEL-28 cells.** To determine the effects of DHM treatment on cell cycle distribution in SK-MEL-28 cells, the previously described procedure was performed with slight modification (14). Briefly, cells were treated without or with 50 and 100 µM DHM for 24, 48 and 72 h. Subsequently, the cells were digested with 0.25% trypsin-EDTA solution and centrifuged at 1X1,000 rpm. The harvested cells were pipetted into 75% ethanol for 1 h, and were centrifuged to discard the 75% ethanol. The pellet of cells was re-hydrate for 15 min at room temperature. Subsequently, the cell suspension was centrifugated to discard the supernatant. The cells were incubated with cell cycle staining solution containing propidium iodide for 30 min at room temperature according to the operating instructions. Then, stained cells were measured by FACSscan (Becton-Dickinson, Franklin Lakes, NJ, USA) for cell cycle distribution, and the experiments were carried out in duplicate for three times.

**TUNEL assay for detecting apoptosis induced by DHM.** The fragmentation of nuclear chromatin is one of the important hallmarks of late-stage apoptosis. Thus, in order to detect the apoptosis of SK-MEL-28 cells treated by DHM, TUNEL assay was used to measure nuclear DNA fragmentation according to the instructions provided in the kit. Briefly, SK-MEL-28 cells were treated with various final concentrations of 0 (served as control), 50 and 100 µM DHM after cell inoculation and were seeded in glass bottom microwell dishes (MatTek Corp., Ashland, MA, USA). After 48 h of DHM treatment, the culture medium was removed, and the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min at 4°C. Subsequently, the cells were pretreated with 0.2% Triton X-100 in PBS for 5 min, and stained for 1 h at 37°C with compound solution, consisting of fluorescein-12-dUTP, dATP, Tris-HCl, EDTA and terminal deoxynucleotidyl transferase according to the instructions, and then were counterstained with DAPI (blue) for 5 min at room temperature. The cells were observed under a laser confocal scanning microscope (Leica TCS SP8; Leica Microsystems Mannheim, Germany) using a standard parameter for green fluorescence of fluorescein. To measure the apoptotic cells, at least five images were randomly captured for the control and treated cells, respectively.

**Western blot analysis.** Human melanoma SK-MEL-28 cells were treated with 50 and 100 µM DHM for 24 and 48 h, and cell lysates were prepared in lysis buffer [0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 1% NP-40, 0.02% NaN₃, 1 mM sodium orthovanadate, 2.5% deoxycholic acid, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail set (Roche, Penzberg, Germany)]. The lysates were centrifuged at 12,000 x g at 4°C for 10 min to obtain supernatant soluble proteins, and the protein concentrations were determined using the BCA assay (Beyotime Institute of Biotechnology, Beijing, China). Equal amounts of protein (10 µg) from each sample were separated by electrophoresis on 10% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride (PVDF) membranes, and then the PVDF membranes were blocked with 5% skim milk in 1X TBST containing 0.1% Tween-20 at room temperature for 1 h. The membranes were probed with primary antibodies including p53, p21, Cdc25A, Cdc2, P-Cdc2, Bax, IKK-α, NF-xB p65, p38 and P-p38 as well as β-tubulin as a loading control. The membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The bands were detected by an enhanced chemiluminescence reagent and system (Amersham Biosciences Corp., Piscataway, NJ, USA).

**Statistical analysis.** Data are expressed as means ± SD. Statistical analysis was performed by the Student's t-test, and
a probability (P) value of <0.05 was considered to indicate a statistically significant result.

Results

DHM exerts a growth inhibitory effect on SK-MEL-28 cells. The inhibitory effect of DHM on cell proliferation was evaluated by MTT assay after incubation with several concentrations of DHM for 24, 48 and 72 h. The results indicated that DHM treatment for 48 and 72 h, had a strong growth inhibitory effect on SK-MEL-28 cells in a concentration- and time-dependent manner (Fig. 1B).

DHM induces cell cycle arrest in SK-MEL-28 cells. It is well known that the most common phenotype observed in cancer cells is their rapid proliferation rate, which causes the cell cycle to be susceptible to modulation. In order to determine whether DHM has any effects on cell cycle distribution of SK-MEL-28 cells, we tested the cell cycle status by using flow cytometry after cells were treated with 50 and 100 µM DHM for 24, 48 and 72 h. The results showed that DHM treatment for 24 h did not significantly change the cell cycle profile in the SK-MEL-28 cells, whereas DHM treatment induced a significant increase in the percentage of cells in the G1 phase with a parallel significant reduction in cells in the S and G2/M phase following 48 h in SK-MEL-28 cells, compared to the control (Table I). Furthermore, continuous DHM treatment for up to 72 h led to a high increase in the proportion of G1 phase cells, causing a reduction in cells in the S and G2/M phase of the cell cycle in SK-MEL-28 cells. These results suggest a potential blockade of cell cycle progression induced by DHM at the G1/S checkpoint in SK-MEL-28 cells.

Since it has been well recognized that the tumor-suppressor gene p53 regulates the expression and activity of downstream genes that control cell cycle progression, we decided to determine the expression level of p53 and the cell cycle-related biomarkers, p21, cell division cycle 25A (Cdc25A), cell division cycle 2 (Cdc2) and P-Cdc2. We found that immunoblotting of SK-MEL-28 cell lysates after treatment with 50 or 100 µM DHM for 48 h demonstrated an apparent increase in

Table I. Effects of DHM treatment on cell cycle distribution of SK-MEL-28 cells.

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration (µM)</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>0 53.23±0.56</td>
<td>32.93±0.68</td>
<td>13.84±0.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 53.12±0.54</td>
<td>32.21±0.39</td>
<td>14.67±0.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 52.73±0.91</td>
<td>32.52±0.77</td>
<td>14.75±0.74</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>0 54.18±1.32</td>
<td>30.68±0.89</td>
<td>15.14±0.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 59.69±1.65a</td>
<td>26.82±1.52a</td>
<td>13.49±0.66a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 61.34±0.74a</td>
<td>25.71±0.52a</td>
<td>12.94±0.54a</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>0 59.29±2.58</td>
<td>27.73±1.40</td>
<td>12.98±1.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 66.40±3.10a</td>
<td>21.33±2.50a</td>
<td>12.27±0.79a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 73.50±3.05ab</td>
<td>18.13±2.46ab</td>
<td>8.37±0.60ab</td>
<td></td>
</tr>
</tbody>
</table>

SK-MEL-28 cells treated with (50 and 100 µM) or without dihydromyricetin (DHM) for 24, 28 and 72 h were collected and stained with propidium iodide and analyzed using flow cytometry. *P<0.05, significant difference compared to the control; **P<0.05, significant difference compared to SK-MEL-28 cells treated with 50 µM DHM. Statistical analysis was analyzed by one-way ANOVA (n=3 independent experiments).
p53 protein, although DHM did not appear to upregulate p53 expression within 24 h of treatment (Fig. 2). As p21 is one of the most vital downstream transcriptional targets of p53, we also detected the production of p21 protein. Cell numbers in at least five images from each sample were counted. *P<0.05 indicates significant difference compared to the control.

**DHM induces apoptosis in SK-MEL-28 cells.** Cell cycle checkpoint is an important intersection for cell survival or cell death. If conditions where cells live are favorable for successful interphase and mitosis, cells can divide and maintain growth or cells may ‘switch on’ a death procedure. Thus, according to the above results showing cell cycle arrest mediated by DHM, we applied TUNEL analysis to detect the effects of DHM treatment on apoptotic induction in SK-MEL-28 cells. The cells were treated for 48 h at a concentration of 50 or 100 µM DHM, respectively. Following the procedure for TUNEL assay, the stained cells were observed by laser confocal scanning microscopy (Fig. 3A). The results demonstrated that the number of TUNEL-positive cells (apoptotic cells) was significantly increased after 50 and 100 µM DHM treatment for 48 h. Compared to the control, the percentage of positive apoptotic cells increased from 9.50±4.04 to 22.20±2.35 and 27.65±7.65%, respectively after 48 h of DHM treatment at 50 and 100 µM (Fig. 3B). Additionally, the data indicate that nuclear chromatin in SK-MEL-28 cells was fragmented, which is an apoptotic characteristic.

**DHM induces apoptosis by regulating the expression levels of p53, Bax, NF-κB p65, IKK-α and P-p38.** p53 is a critical mediator of cell death and its role in apoptosis is well established (23). Therefore, we assessed the expression levels of p53 downstream genes such as Bax and NF-κB p65. The immunoblot results showed that the expression level of Bax protein in SK-MEL-28 cells was increased after 48 h of DHM treatment, although 24 h of incubation with 50 or 100 µM DHM barely affected its expression level (Fig. 4). In addition, the protein expression level of NF-κB p65 was decreased within 24 or 48 h of DHM treatment. We also assessed the expression levels of upstream protein, IKK-α. The results showed that IKK-α protein was apparently downregulated between 24 and 48 h of incubation with 100 µM DHM. Unexpectedly, DHM treatment for 24 h decreased its protein expression, but
continuous treatment for up to 48 h upregulated its expression. Moreover, we found that although the protein level of p38 was slightly affected, its phosphorylated form, P-p38, was clearly downregulated in a concentration- and time-dependent manner (Fig. 4). Taken together, DHM treatment led to upregulation of p53 and Bax protein expression, and decreased expression levels of NF-κB p65, IKK-α and P-p38, which were associated with DHM-induced apoptosis in SK-MEL-28 cells.

Discussion

Melanoma is one of the most aggressive forms of cancer with increasing incidence rates and high resistance to current therapies (1,24). Until recently, no agent has been developed for the effective long-term treatment of patients with metastatic skin cancer (25,26). With so few treatment options available, new therapeutic agents are urgently needed to prevent and overcome this aggressive cancer. Therefore, to investigate a possible anti-melanoma effect of DHM as a first step toward the development of a novel putative anticancer agent, we studied DHM for its capability to inhibit cell proliferation in the melanoma SK-MEL-28 cell line.

Previous reports showed that DHM is able to inhibit cell growth in a panel of cancer cell lines, such as human prostate cancer cell lines PC-3 and LNCaP (14), human bladder carcinoma EJ cells and murine sarcoma 180 cells (15), and hepatoma HepG2 cell line (17). In the present study, we firstly found that DHM significantly inhibited the proliferation of the human melanoma cell line SK-MEL-28 (Fig. 1B). Furthermore, our data demonstrated that the antiproliferation effect of DHM in SK-MEL-28 cells was associated with cell cycle arrest and induction of apoptosis.

We confirmed that DHM treatment arrested SK-MEL-28 cells at the G1 phase, as well as decreased the proportion of cells at the S and G2/M phase (Table I). Notably, a previous study reported that DHM induced cell cycle arrest at the S phase in LNCaP cells or at the S and G2/M phase in PC-3 cells (14). We estimated that the mechanism involved in cell cycle arrest caused by DHM varied in different cancer cells. Furthermore, to clarify the mechanism of DHM-mediated cell cycle arrest in melanoma SK-MEL-28 cells, we confirmed the hypothesis that DHM modulates the p53 signaling pathway to affect cell cycle status. The results revealed a significant increase in p53 protein expression after 48 h of DHM treatment (Fig. 2). p53 functions as a node for organizing whether cells respond to various types and levels of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism or autophagy (27). Serving as one of the most important p53 target genes inducing growth arrest, expression level of p53 protein in the SK-MEL-28 cells was barely detectable compared with the healthy melanoma SK-MEL-28 cells (Fig. 1B). Furthermore, our data demonstrated that the antiproliferation effect of DHM in SK-MEL-28 cells was associated with cell cycle arrest and induction of apoptosis.

Following DHM treatment, the proportion of S and G2/M phase cells via upregulation of the p53 and p21 protein expression in SK-MEL-28 cells. However, it is still not clear whether DHM treatment causes translocation or a change in the subcellular localization of p21, and this issue requires further study. The primary substrate of Cdc25A is cyclin-dependent kinase 2 which allows cell cycle progression through the G1/S checkpoint when active. Our data indicated that expression levels of Cdc25A, Cdc2 and P-Cdc2 were decreased, similar to previous studies (31-33).

The induction of apoptosis is considered to be a useful approach for cancer therapy. The MTT assay initially indicated that DHM acts as an antiproliferation agent against melanoma SK-MEL-28 cells (Fig. 1B). Therefore, we investigated whether DHM-mediated growth arrest was due to induction of apoptosis apart from cell cycle arrest. In our experiments, significant apoptosis following DHM treatment was observed by laser confocal scanning microscopy (Fig. 3). Further investigation indicated that apoptosis induced by DHM was associated with the upregulation of expression levels of p53 and Bax protein (Fig. 4). p53-mediated apoptosis has been intensively studied since it was first demonstrated (34). Numerous publications have confirmed the importance of p53 transcriptional regulation in apoptosis. Bax is one of the p53 target genes inducing apoptosis, and so is an important regulator of apoptosis (35). p53 interacts with Bcl-XL or Bcl-2 to promote the oligomerization of Bak and Bax that assemble in the mitochondrial membrane to form pores, resulting in the release of cytochrome c and other apoptotic activators from the mitochondria (5,36). Overexpression of Bcl-2 blocks p53-mediated apoptosis, whereas, Bax binds to the Bcl-2 protein and abolishes its anti-apoptotic ability. In brief, the p53-dependent increase in Bax leads to apoptosis. Moreover, we did not detect the expression level of Bcl-2 protein in the SK-MEL-28 cells by western blot analysis. It has been reported that the expression level of Bcl-2 protein in SK-MEL-28 cells was barely detectable compared with the induction of Mcl-1S and Bak protein after 18 h of exposure to bortezomib, a proteasome inhibitor (37). In the present study, the expression levels of NF-κB p65 and its upstream IKK-α protein were downregulated following DHM treatment. NF-κB regulates cell proliferation and survival in eukaryotic cells. Active NF-κB turns on the expression of genes which promotes cell proliferation and protects cells from apoptosis. It was found that blockage or defective NF-κB can make tumor cells stop proliferating and enhance the susceptibility to apoptosis (38). We hypothesized that downregulation of
NF-κB p65 probably increased the sensitivity of SK-MEL-28 cells to DHM and contributed to induction of apoptosis. IKK-α is an upstream regulatory component for the NF-κB signaling pathway (38). A decline in the expression level of IKK-α possibly boosts the downregulation of NF-κB p65 and helps to strengthen anticancer ability. In the present study, we found that the expression level of p38 in SK-MEL-28 cells was hardly affected after 24 or 48 h of incubation, while the expression level of P-p38 was unexpectedly decreased (Fig. 4). The signaling of mitogen-activated protein kinases (MAPks) regulates various cellular functions such as cell differentiation, proliferation and cell death. p38 is one component of the MAPK family, and p38 activation generally possesses a pro-apoptotic function. A previous study showed that Cdc25B degradation was dependent mainly on c-Jun N-terminal kinase (JNK) and partially on p38 (39). We hypothesized that down-regulation of P-p38 in SK-MEL-28 cells probably contributed to pro-apoptotic activity and cell cycle arrest. In addition, we also found that the phosphorylated form of p38 was decreased in hepatoma HepG2 cells (unpublished data). The effects of DHM treatment on the signaling of MAPks consisting of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 and extracellular signal-regulated kinase (ERK1/2) still require further clarification. Taken together, the mechanisms of DHM that induced cell cycle arrest and apoptosis in SK-MEL-28 cells are possibly complex (Fig. 5).

In conclusion, our results demonstrated the inhibitory effects and induction of cell cycle arrest at the G1 phase and apoptosis in SK-MEL-28 cells in response to DHM treatment. Within the above indicated dosage range, DHM treatment upregulated the production of p53 protein, including its downstream transcriptional target genes such as p21 and Bax protein in SK-MEL-28 cells. Moreover, DHM also down-regulated the protein level of NF-κB p65, IKK-α and P-p38. Although, these data apparently imply a pivotal role of p53 in DHM-mediated cell cycle arrest and apoptosis, additional research must be performed to clarify the role of p53 upstream and downstream signal networks. In summary, our results imply that DHM treatment may be a new therapeutic strategy against the growth of melanoma.

Acknowledgements

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