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7,8-Dimethoxycoumarin stimulates melanogenesis via MAPKs mediated MITF upregulation

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Background: Melanin in the skin is the defense against the harmful UV radiation, which is considered as one of the major risk factors for skin cancer. The compound 7,8-dimethoxycoumarin (DMC, $C_{11}H_{10}O_4$), a natural coumarin molecule present in several medicinal plants, possesses antioxidant and anti-inflammatory activities. However, the mechanism underlying its effects on melanogenesis in melanocytes is unclear. Therefore, we investigated the effect of DMC on melanogenesis activation in B16F10 melanoma cells. *Methods*: We examined the cytotoxic range of DMC on B16F10 melanoma cells and increased effects of melanogenesis, and intracellular tyrosinase activity. In addition, regulation mechanisms were assessed by Western blot analysis. *Results*: The results showed that DMC significantly increased melanin content and tyrosinase activity in the cells without being cytotoxic. Furthermore, DMC stimulated the expression of tyrosinase, TRP-1, TRP-2, and MITF thereby activating melanin production and Akt phosphorylation was increased in the Akt signaling pathway. on the contrary, interfering with the phosphorylation of ERK in the MAPKs pathway. *Conclusions*: These results suggest that DMC may serve as a candidate for potential melanin-producing activator and anti-gray hair applications.

1. Introduction

Melanin determines the color of human skin, hair, and eyes, and plays a photoprotective role by absorbing free radicals from the cytoplasm and acting as a shield against ultraviolet (UV) radiation. Melanin is synthesized within unique organelles of melanocytes called melanosomes and transferred to adjacent keratinocytes (Brenner and Hearing 2008; Miyamura et al. 2007; Liu et al. 2005). Melanin synthesis is mainly regulated by melanogenic enzymes such as tyrosinase (TYR, EC 1.14.18.1), tyrosinase-related protein 1 (TRP-1, 5,6-dihydroxyindole-2-carboxylic acid oxidase, EC 1.14.18), and tyrosinase-related protein 2 (TRP-2, dopachrome isomerase, EC 5.3.3.12). At transcription level, the expression of melanogenic enzymes is upregulated by the binding of microphthalmia-associated transcription factor (MITF), which is involved in pigmentation, proliferation, and survival of melanocytes (Tsukamoto et al. 1992; Stevens et al. 1998; Barton et al. 1988). MITF also binds to the M-box within the tyrosinase promoter following the upregulation of tyrosinase gene expression. Extracellular signal-regulated kinases (ERK), in turn, potently induce the expression of MITF and increase melanin synthesis (Vachtenheim et al. 2013, 2010). Abnormal pigmentation conditions may be divided into two types as follows: hypermelanosis and hypomelanosis. Hypermelanosis refers to a state of excessive melanin synthesis in the skin, while hypomelanosis is a state characterized by insufficient melanin content in the skin (Bauer et al. 2003; Saleem et al. 2019; Slominski et al. 2004; Hearing and Jiminez 1987). Several extrinsic and intrinsic factors are involved in the synthesis of melanin through intracellular signaling pathways, such as mitogen-activated protein kinases (MAPKs), cyclic adenosine 3′,5′-monophosphate (cAMP), and protein kinase C (PKC). (Lee et al. 2018; Ahn et al. 2008; Kang et al. 2011; Saha et al. 2006)

Coumarins belong to the family of benzopyrones and comprise a benzene ring joined by a pyrone ring. Coumarins have been described to exhibit anticoagulant, bacteriostatic, antitumor, anti-aging, and cardioprotective properties. The isolation, structural characterization, biosynthetic pathway, and biological activity of thousands of natural coumarins from plants, bacteria, and fungi as well as those chemically synthesized have been studied well. Hence, coumarins occupy an important place in the field of natural products and organic chemistry (Kaur et al. 2015).

The compound 7,8-dimethoxycoumarin (dimethoxychromen-2-one, DMC) (Fig. 1a) is a natural coumarin molecule present in several medicinal plants, including *Daphne koreana*, *Astianthus viminalis*, *Zanthoxylum leprieurii*, and *Citrus decumana*. DMC was shown to protect the kidney against cisplatin and ischemia-reperfusion injury *via* antioxidant, anti-inflammatory activities as well as through the inactivation of the opening of mitochondrial permeability transition pore. DMC, isolated from the ethyl acetate extract of *C. decumana*, also showed anti-secretary and anti-inflammatory activities on pyloric ligation-induced gastritis in rats (Huang et al. 1990; Pérez Gutiérrez et al. 2009; Sood et al. 2010; Muthuraman et al. 2012; Misra et al. 2010). However, the effects against melanogenesis and hypopigmentation and the basic mechanism of action of DMC are unclear so far. Therefore, as a part of a prominent research program to develop novel substances for nutraceutical and cosmetic purposes, we examined the effect of DMC on melanin production and melanogenic enzyme expression and investigated the molecular mechanism underlying these effects in B16F10 mouse skin melanoma cells.

Recent studies have shown that benzo-gamma-pyrones represented by flavonoids with methoxy structures are more useful in functional and industrial aspects than the flavonoids with hydroxy structures. We have previously reported that polymethoxy flavonoids (PMFs) such as tangeretin and sinensetin may effectively stimulate melanogenesis through an increase in melanin production via MAPK signaling pathway (Yoon et al. 2015 a,b;; Chung et al. 2018). Therefore, we determined the effect of PMFs to evaluate the specific mechanism underlying DMC-regulated activation of melanin synthesis and investigated the effect of DMC on signal

transduction pathways. MAPK and protein kinase B (AKT) signaling pathways are known to be involved in the activation or inhibition of melanogenesis. ERK activation is responsible for the regulation of MITF protein stability through its phosphorylation. Increased phosphorylation of MITF results in a decrease in protein stability and leads to its degradation in proteasomes. Therefore, we investigated the mechanism underlying DMC-induced melanogenesis in B16F10 cells. DMC treatment increased the phosphorylation of AKT in a concentration-dependent manner as opposed to the dramatic reduction in the phosphorylation of ERK. The involvement of other MAPK pathways was also examined and a significant phosphorylation level of JNK and p-38 MAPK was observed.

2. Investigations and results

2.1. Effect of DMC on melanin production and the viability of B16F10 cells

In the present study, murine B16F10 melanoma cells were used as the cell model to examine the effect of DMC on melanogenesis. To evaluate the cytotoxic effects of DMC on B16F10 cells, MTT assay was performed. B16F10 cells were treated with various concentrations (0.05, 0.1, 0.2, 0.4, and 0.8 mM) of DMC for 72 h. As shown in Fig. 1b, no significant difference was observed in cell proliferation between control and DMC-treated cells up to 0.4 mM concentration of DMC. Given the low cytotoxic effect of DMC on B16F10 cells, the stimulatory effects of DMC on melanin synthesis were assessed. In comparison with α-MSH-treated (100 nM) groups, DMC-treated (0.025, 0.05, 0.1, 0.2, and 0.4 mM) groups showed an increase in melanin production at 72 h in a dose-dependent manner (Fig. 1c).

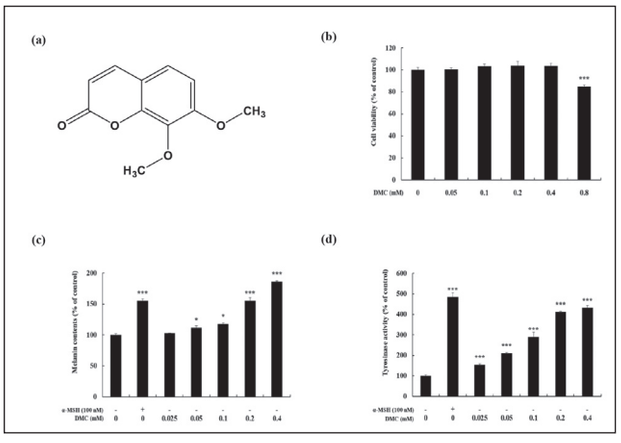

Fig. 1: Effects of DMC on cell viability, melanin content, and Intracellular tyrosinase activity in B16F10 cells. (a) Structure of 7, 8-dimethoxycoumarin (DMC). (b) The cell viability test that cells were treated with DMC (0.05, 0.1, 0.2, 0.4, and 0.8 mM) for 72 h. (c) The melanin content and (d) tyrosinase activity was that cells were treated with DMC (0.025, 0.05, 0.1, 0.2, and 0.4 mM) for 72 h. α-MSH (100 nM) was used as the positive control. The data are expressed as percentages compared to the respective values obtained for the untreated cells and presented as mean ± standard deviation (SD) of at least three independent experiments (n=3). * $p < 0.05$, *** $p < 0.001$ vs. control.

2.2. Effect of DMC on intracellular tyrosinase activity in B16F10 cells

Tyrosinase plays a very important role in the biosynthesis of melanin by catalyzing the oxidation of L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA) to dopaquinone. Dopaquinone thus formed undergoes intramolecular cyclization to form leucodopachrome, which is further oxidized to dopachrome (Hearing and Jiminez 1987). Tyrosine ultimately regulates the production of pigments in the epidermal melanocytes. In this regard, the melanogenic capability of DMC was evaluated in terms of its effect on intracellular tyrosinase in B16 melanocytes. We pretreated B16F10 cells with DMC at various doses (0.025, 0.05, 0.1, 0.2, and 0.4 mM). As shown in Fig. 1d, DMC treatment significantly increased the cellular tyrosinase activity in a dose-dependent manner as compared to the control. Furthermore, DMC had no inhibitory activity on mushroom tyrosinase (data not shown).

2.3. Effect of DMC on the protein expression of melanogenic enzymes in B16F10 cells

The expression of melanogenic enzymes such as tyrosinase, TRP-1, and TRP-2 is regulated by MITF, a major transcription factor involved in the process of melanogenesis (Chung et al. 2017). To investigate whether DMC influences melanogenic protein expression, western blot analysis was performed using the lysate from DMC-treated B16F10 cells. As a result, the expression of tyrosinase, TRP-1 and TRP-2 protein was significantly increased (Fig. 2).

As shown in Fig. 3, the increases of these melanogenic enzymes can be seen to be caused by an increase in MITF expression. The protein level of β-actin, a housekeeping protein used as an internal control, showed no change. These results suggest that MITF protein levels increase following DMC treatment. The hyperpigmentation effect of DMC may be associated with the upregulation in MITF gene expression, which would eventually activate melanogenic protein expression.

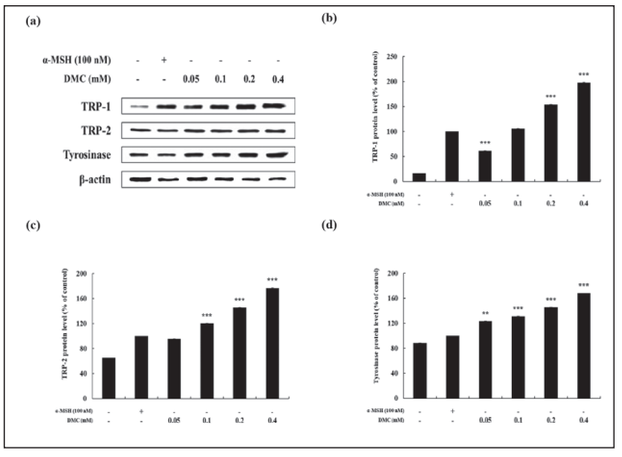

Fig. 2: Effects of DMC on TRP-1, TRP-2, and Tyrosinase expression in B16F10 cells. The cells were treated with the indicated concentrations of DMC for 40 h. α-MSH (100 nM) was used as the positive control. Protein levels were determined by western blotting. (**a**) Western blotting results and protein levels of (**b**) TRP-1, (**c**) TRP-2, and (**d**) Tyrosinase. Results are expressed as percentages of the positive control. The data are presented as the mean ± SD of at least three independent experiments (n=3). ** $p < 0.01$, *** $p < 0.001$. vs. positive control.

2.4. Effect of DMC on the protein expression of MITF in B16F10 cells

To determine the mechanism underlying DMC-regulated activation of melanin synthesis, we investigated the effect of DMC on signal transduction pathways. The activation of MAPK and AKT signaling pathways has been reported to be involved in activating or inhibiting melanogenesis. ERK activation is responsible for the regulation of MITF protein stability through its phosphorylation. Increased phosphorylation of MITF results in a decrease in the enzyme stability and leads to degradation in proteasomes (Jang et al. 2017; Sim et al. 2017). Therefore, we investigated the effects of DMC on the increased expression of MITF regulator are responsible for MAPK and PI3K/AKT phosphorylation in B16F10 cells.

2.5. Effect of DMC on the AKT, MAKP signaling pathway in B16F10 cells

As shown in Figs. 4 and 5, DMC treatment increased the phosphorylation of AKT in a concentration-dependent manner as opposed to the dramatic reduction in the phosphorylation of ERK.

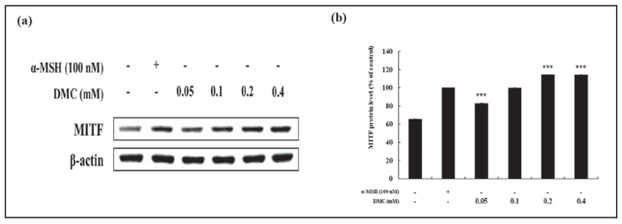

Fig. 3: Effects of DMC on MITF expression in B16F10 cells. The cells were treated with the indicated concentrations of DMC for 24 h. α-MSH (100 nM) was used as the positive control. Protein levels were determined by western blotting. (a) Western blotting results and protein levels of (b) MITF. Results are expressed as percentages of the positive control. The data are presented as the mean ± SD of at least three independent experiments (n=3). ** $p < 0.01$, *** $p < 0.001$ vs. positive control.

And we also evaluated the involvement of other MAPK pathways and found significant activation in phosphorylation of p38, and JNK. Additionally, we further investigated the mechanism of the DMC-induced melanogenesis in B16F10 cells (Chae et al. 2017).

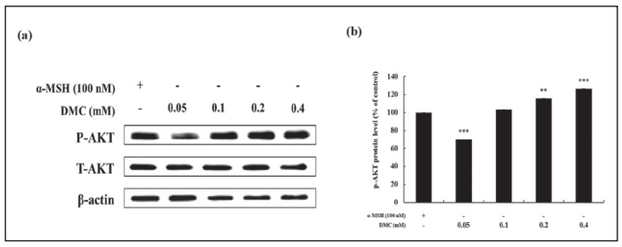

Fig. 4: Effects of DMC on AKT signaling pathway in B16F10 cells. The cells were treated with the indicated concentrations of DMC for 4 h. α-MSH (100 nM) was used as the positive control. Protein levels were determined by western blotting. (a) Western blotting results and protein levels of (b) AKT. Results are expressed as percentages of the positive control. The data are presented as the mean ± SD of at least three independent experiments (n=3). *** $p < 0.001$ vs. positive control.

2.6. Effect of ERK inhibitor on ERK signaling pathway and tyrosinase activity in B16F10 cells

We evaluated the expression of the MAPK signaling pathway in B16F10 cells co-treated with DMC, and ERK inhibitor (PD98059). As shown in Fig. 6a and 6b, the DMC reduced the phosphorylation of ERK to the level observed after α-MSH treatment. In particular, phosphorylation further reduced following co-treatment of cells

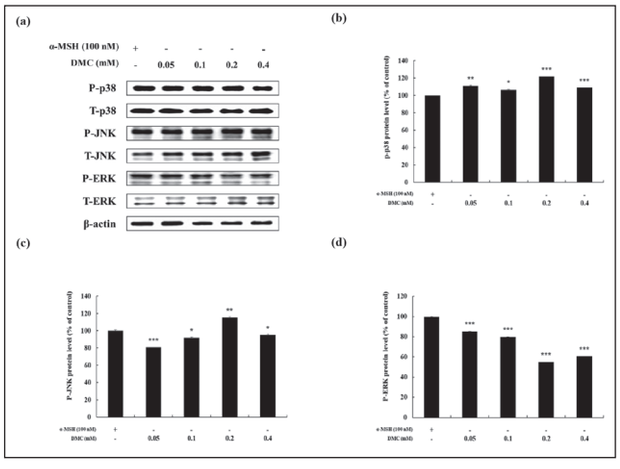

Fig. 5: Effects of DMC on MAPKs signaling pathway in B16F10 cells. The cells were treated with the indicated concentrations of DMC for 4 h. α-MSH (100 nM) was used as the positive control. Protein levels were determined by western blotting. (a) Western blotting results and protein levels of (b) P-p38, (c) P-JNK, and (d) P-ERK. Results are expressed as percentages of the positive control. The data are presented as the mean ± SD of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. positive control.

with DMC and PD98059. However, no significant change in the phosphorylation of p38 and JNK was observed (data not shown). These findings indicate that the stimulatory effects of DMC against melanogenesis were associated with the inhibition of ERK pathway (Kim et al. 2003).

Furthermore, tyrosinase activity was measured after treating cells with ERK inhibitor to determine whether the decrease in ERK phosphorylation resulted in the upregulation in the intracellular tyrosinase activity of B16F10 cells. In comparison with the cells from the negative control group, PD98059-treated cells showed an increase in tyrosinase activity following DMC treatment (Fig. 6c). These results demonstrate that DMC induces upregulation in tyrosinase expression in association with ERK pathway.

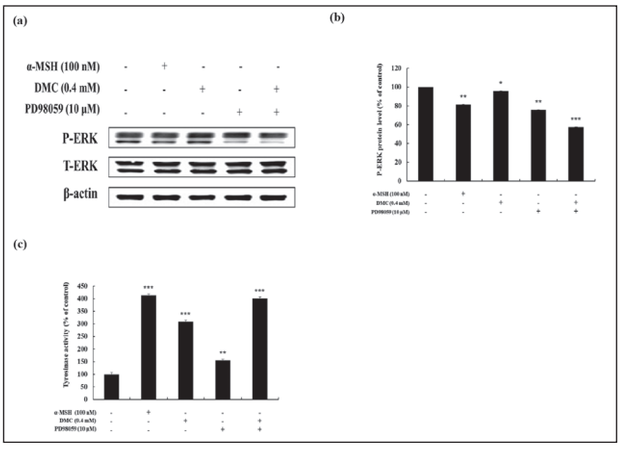

Fig. 6: Effects of ERK inhibitor PD98059 on ERK pathway and tyrosinase activity in B16F10 cells. The cells were treated with ERK inhibitor PD98059 (10 μM) alone or in combination with DMC (0.4 mM). α-MSH (100 nM) was used as the positive control. Protein levels were determined by western blotting. (a) Western blotting results and protein levels of (b) P-ERK, and (c) results of tyrosinase activity. Results are expressed as percentages of the control. The data are presented as the mean ± SD of at least three independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

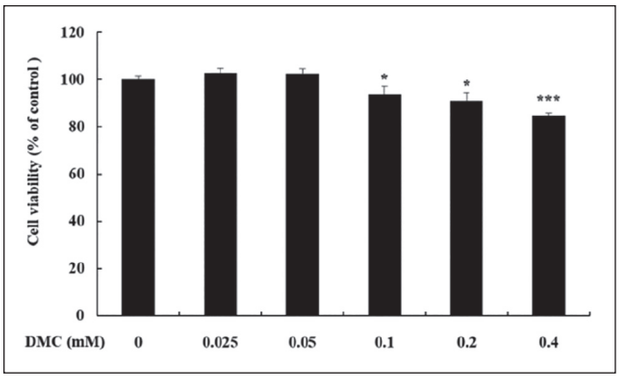

Fig. 7: Effect of DMC on the viability of HaCaT cells. Cells were treated with DMC (0.025, 0.05, 0.1, 0.2, and 0.4 mM) for 20 h. Data are presented as mean ± standard deviation (SD) of at least four independent experiments (n=4). * $p < 0.05$, *** $p < 0.001$ vs. control.

2.7. Effect of DMC on viability of HaCaT cells

Skin is the body's largest organ and serves as the body's first line of defense against infection and regulates temperature and fluid balance. The epidermal layer is made up of keratinocytes with interconnected melanocytes. The melanosomes are produced in melanocytes and are transmitted to keratinocytes, affecting skin pigmentation and UV radiation blocking (Wang et al. 2016). Therefore we examined the cytotoxic effect of DMC on a human keratinocyte HaCaT cell line to evaluate the safety associated with the application of DMC as a topical agent. As shown in Fig. 10, cell viability was almost 91% following exposure to DMC

at concentrations below 0.2 mM. These data suggest that DMC exhibits low cytotoxicity against mammalian cell lines.

3. Discussion

In this study, we evaluated the effect of 7,8-dimethoxycoumarin on melanogenesis mechanism in B16F10 mouse melanoma cells. Our results confirmed that melanogenesis and tyrosinase activity were increased in a dose-dependent manner (0.025, 0.05, 0.1, 0.2, and 0.4 mM) within the range of no cytotoxicity of DMC (Fig. 1). To determine the mechanism underlying DMC-regulated activation of melanin synthesis we investigated the effect of DMC on signal transduction pathways by western blot analysis. This melanogenesis was confirmed through the expression of melanogenic enzymes. As expected, our results showed that DMC induced melanogenesis in melanin-producing enzymes (tyrosinase, TRP-1, and TRP-2) on B16F10 cells after 40 h in a dose-dependent manner through western blot experiments (Fig. 2). These melanin-producing enzymes are transcriptionally regulated by MITF. So, in order to evaluate whether DMC affects the MITF regulator, we conducted a western blot analysis. Our results showed that DMC-induced increase in MITF expression reached a maximum level after 24 h of treatment, is also higher than that of α-MSH, which is positive control (Fig. 3). Thus, it can be seen that DMC increases melanin production by increasing MITF gene expression. Previous studies have shown that transcription of MITF can be regulated by modulation of phosphorylation of AKT in the cAMP pathway. The increase in AKT phosphorylation induces the accumulation of β-catenin through phosphorylation of GSK-3β in ser21/9, and β-catenin accumulated in the cytoplasm increases the expression of MITF by translocation to the nucleus (Su et al. 2013).

As shown in Fig. 4, in the AKT pathway DMC increased phosphorylation of AKT in a concentration-dependent manner compared to α-MSH. These results show that DMC induces melanogenesis through increased p-AKT. However, exploring the precise sub-mechanism may require further experiments. The MAPKs signaling pathway is known to regulate the transcriptional activity of MITF *via* direct phosphorylation. And activation of ERK MAP kinases diminishes the tyrosinase activity and melanin production in B16F10 cells (Lee et al. 2018). In this study, the western blot assay showed that DMC significantly decreased the expression levels of p-ERK but p-p38 and p-JNK was increased in B16F10 cells after 4 h (Fig. 5). Therefore, we evaluated the expression of the MAPK signaling pathway in B16F10 cells co-treated with DMC and ERK inhibitor (PD98059) by western blot and tyrosinase activity. As shown in Figs. 6a and 6b, DMC reduced the phosphorylation of ERK to the level observed after α-MSH treatment. In particular, phosphorylation further reduced following co-treatment of cells with DMC and PD98059 than α-MSH treated control. However, no significant change in the phosphorylation of p38 and JNK was observed (data not shown). In addition, compared with the cells from the negative control group, PD98059-treated cells showed an increase in tyrosinase activity following DMC treatment (Fig. 6c). These findings indicate that the stimulatory effects of DMC against melanogenesis were associated with the inhibition of the ERK pathway.

In conclusion, the present study describes for the first time the stimulatory effects of DMC on melanogenesis in B16F10 melanoma cells. DMC increased melanin content in B16F10 cells by upregulating MITF expression and interfering with ERK phosphorylation. These results suggest that DMC provides evidence for its possible use for anti-gray hair applications and as a therapeutic agent against hypopigmentation disorders.

4. Experimental

4.1. Chemicals and reagents

All solvents were of analytical grade and used without any further purification. 7,8-Dimethoxycoumarin (DMC, $C_{11}H_{10}O_4$) and α-melanocyte stimulating hormone (α-MSH), sodium hydroxide (NaOH), and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MI, U.S.A). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from VWR (Radnor, Pennsylvania, U.S.A). trypsin-EDTA solution, BCA protein assay kit, and PD98059 (ERK inhibitor) were purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A). Antibodies against TRP-1, TRP-2, Tyrosinase, MITF, and SP600125 (JNK inhibitor) were purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A) and P-p38, T-p38, P-JNK, T-JNK, P-ERK, T-ERK, β-actin were purchased from Cell Signaling Technologies (Danvers, MA, U.S.A). SB203580 (p38 inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A). dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), radioimmunoprecipitation (RIPA) buffer, enhanced chemiluminescence (ECL) kit were purchased from Biosesang (Sungnam, Gyeonggi-do, Korea) and 2 laemmli sample buffer were purchased from Bio-Rad (Hercules, CA, U.S.A).

4.2. Cell culture

B16F10 murine melanoma cells were maintained in Dulbecco's modified eagle medium (DMEM, phenol red-free) supplemented with 10% fetal bovine serum (FBS, Merck KGaA, Darmstadt, Germany) and 1% penicillin-streptomycin (5000 units/mL and 5000 μg/mL respectively). The cells were maintained in a humid atmosphere of 5% CO_2 at 37 °C.

4.3. Cell viability assay

The viability of B16F10 and HaCaT cells was determined using MTT colorimetric assay (Gerlier and Thomasser 1986). This assay is based on the ability of succinic dehydrogenase synthesized by living cells to reduce the yellow MTT salt to a purple-blue insoluble formazan precipitate. The signal generated is dependent on the activity status of the cells. Briefly, B16F10 cells were seeded in a 24-well plate at 2×10^4 cells/well and pre-incubated for 24 h. The supernatant was removed and fresh media with DMC (0.05, 0.1, 0.2, 0.4, and 0.8 mM) were added to each well. The cells were cultured for 72 h. HaCaT cells were seeded in a 24-well plate at 1.5×10^5 cells/mL and pre-incubated for 24 h. The supernatant was removed and DMC (0.025, 0.05, 0.1, 0.2, and 0.4 mM) was added to the medium without FBS and the cells were cultured for 20 h. After incubation, the cell culture supernatant was removed and 400 mL of 0.2 mg/mL MTT solution was added to each well. Following incubation for 3 h, the MTT solution was removed and the formazan crystals formed were dissolved by adding DMSO. The absorbance of the solution was measured at 570 nm using a microplate reader. The effect of DMC on cell viability was evaluated by comparing the absorbance of the test to that of the untreated control.

4.4. Intracellular melanin content

Melanin content in the cells was measured using a previously described method with slight modifications (Hu et al. 2008). Briefly, B16F10 cells were seeded in a 60-mm culture dish at 1×10^5 cell/dish for 24 h. The cell supernatant was removed and fresh media with DMC (0.025, 0.05, 0.1, 0.2, and 0.4 mM) or α-MSH (100 nM) were added to each well. The cells were cultured for 72 h. α-MSH was used as the positive control. After incubation, the cultured cells were washed twice with 1 PBS and cell pellets were harvested and dissolved in 200 mL of 1 N NaOH containing 10% DMSO at 70 °C for 1 h. Next, 50 mL aliquots of the media were placed in 96-well plates and the absorbance was measured at 405 nm wavelength using a microplate reader. The protein concentration was determined with BCA protein assay using bovine serum albumin (BSA) as a standard. The melanin content was determined based on the protein concentrations (absorbance/mg protein) in the extract from each cell.

4.5. Intracellular tyrosinase activity

Cellular tyrosinase activity was measured as per the previously described method, with some modifications (Jara et al. 1988). B16F10 cells were plated in a 60-mm culture dish at 1×10^5 cells/dish and pre-incubated for 24 h. The cell supernatant was removed and fresh media with DMC (0.025, 0.05, 0.1, 0.2, and 0.4 mM) or α-MSH (100 nM) were added to each well. The cells were cultured again for 72 h. α-MSH was used as the positive control. After incubation, the cultured cells were washed twice with 1 PBS and lysed in a lysis buffer (150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl pH 7.5, and 2 mM EDTA, sterile solution) and protease inhibitor cocktail (1.0%) at 4 °C for 20 min and centrifuged at 21,055 *g* for 20 min. The protein concentration was quantified using BCA protein assay kit. The protein content in each cell lysate sample was adjusted using 0.1 M sodium phosphate buffer (pH 6.8) to ensure equal protein loading. Next, 20 mL of each lysate and 80 mL of L-DOPA (2 mg/mL) were mixed in the wells of a 96-well plate. After incubation at 37 °C for 2 h, the absorbance was measured at 490 nm wavelength using microplate reader.

4.6. Western blot analysis

B16F10 cells were seeded in a 60-mm culture dish at 1×10^5 cells/dish and pre-incubated for 24 h. The cell supernatant was removed and fresh media with DMC (0.05, 0.1, 0.2, and 0.4 mM) or α-MSH (100 nM) were added to each well. The cells were incubated for different time points depending on the antibody used. After incubation, the cultured cells were washed twice with 1 PBS and lysed with lysis buffer and protease inhibitor cocktail (1.0%) at 4 °C for 20 min and centrifuged at 21,055 *g* for 20 min. The protein concentration was measured using BCA protein assay kit and 20 mg of each protein was loaded onto an SDS polyacrylamide gel electrophoresis (PAGE) gel. The separated bands were transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat skim milk (blocking buffer) dissolved in tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature. The blots were washed thrice with TBS-T and overnight probed with primary antibodies (1:1,000). After completion of the reaction, the blots were

washed five times at 10 min intervals with TBS-T and incubated with a suitable secondary antibody (1:3,000) for 2 h. The membranes were washed four times at 10 min intervals with TBS-T. Proteins were detected using ECL kit and images were captured and analyzed using ChemiDoc (Fusion solo 6S.WL, VILBER LOURMAT, France).

4.7. MAP kinase inhibitor assay

B16F10 cells were seeded in a 60-mm culture dish at 1×10^5 cells/dish. After 24 h incubation, cells were treated with 10 μM SB203580, 5 μM SP600125, and 10 μM PD98059, followed by treatment with 0.4 mM DMC. The cells were incubated for 18 h. To confirm the specific MAPK pathway involved in DMC-induced melanogenesis, western blotting was performed to assess the expression of tyrosinase.

4.8. Statistical analysis

The results of all experiments are expressed as mean±standard deviation (SD; n = 3 or 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistical significance and p-values were calculated using Student's t-test.

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Conflicts of interest: None declared.

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