Synthesis and Melanogenesis Effect of 7,8-Dimethoxy-4-Methylcoumarin via MAPK Signaling-Mediated Microphthalmia-Associated Transcription Factor Upregulation

Min-Sung Kang¹, Sung-Chan Jang¹, Taejin Park¹, Min-Seon Kim², Jin-Soo Park², Won-Jae Chi³, and Seung-Young Kim¹

Abstract
Tyrosinase ultimately controls the melanogenesis rate of the skin, and tanning and haircare products generally induce the activation of tyrosinase. Moreover, various enzymes, including tyrosinase, tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2), mediate melanogenesis in which microphthalmia-associated transcription factor (MITF) is a master regulator. One coumarin family member 7,8-dihydroxy-4-methylcoumarin (DHMC) shows extensive biological activities with beneficial health effects; however, it also induces cytotoxicity and its melanogenic effect has not been reported yet. Therefore, we first synthesized DHMC derivatives via methylation to obtain 7,8-dimethoxy-4-methylcoumarin (DMMC), and investigated the pro- or anti-melanogenic effects of DHMC and DMMC in B16-F10 melanoma cells as well as the underlying mechanism. DHMC showed cytotoxicity at all tested concentrations, whereas DMMC did not reduce cell viability, even at the high concentration. DMMC also drives the significant increase in intracellular melanin and tyrosinase activity. Moreover, DMMC induced MITF expression by significantly increasing tyrosinase activity, which activates the gene expression of TRP1 and TRP2. Western blotting confirmed that DMMC induced the activation of mitogen-activated protein kinase (MAPK) signaling by the phosphorylation of C-Jun N-terminal kinase (JNK), resulting in the increased melanin production and the decreased phosphorylation of protein kinase B. Collectively, this study showed the pro-melanogenic effect of DMMC and its potential as a safe tanning and dyeing agent.

Keywords
methylation, 7,8-dihydroxy-4-methylcoumarin, 7,8-dimethoxy-4-methylcoumarin, melanogenesis, B16-F10 melanoma cell, MAPK pathway, coumarin

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Introduction
Melanin is a polymer that is naturally present in animal and plant systems, which is a pigment responsible for skin color and is comprised of indole and other tyrosine-derived intermediates. Melanogenesis is initiated upon exposure to internal and external stimulators such as ultraviolet (UV) rays, chemicals, and bacteria, which occurs in the melanosomes and organs specialized for melanocytes in the skin.¹,² The expression of melanin is regulated by various cellular mechanisms and the pigment is then transmitted to the surrounding keratinocytes. Melanin can be classified into pheomelanin and eumelanin, with the latter largely responsible for determining the color of mammals.

Three main enzymes (tyrosinase, tyrosinase-related protein-1 [TRP1], and tyrosinase-related protein-2 [TRP2]) play a central role in melanin synthesis. L-tyrosine is a required precursor in the early stages of melanin synthesis, during which it is converted into dihydroxy-l-phenylalanine (l-DOPA) through a hydroxy reaction by tyrosinase. DOPA quinone, an oxidation form of l-DOPA to form DOPA chrome, is subsequently converted to 5,6-dihydroxy-2-indolylcarboxylic acid.
acid (DHICA) by TRP2. Ultimately, indole 5,6-quinone-carboxylic acid is generated from DHICA by TRP1, result in the production of brown/black pigment eumelanin. Tyrosinase is a direct inhibitor of the synthesis of eumelanin and pheomelanin, whereas TRP1 and TRP2 are deeply involved in the synthesis of eumelanin. In mice, it was reported that TRP1 oxidize 5,6-dihydroxyindole-2-carboxylic acid to produce indole-5,6-quinone-2-carboxylic acid, although the activity of this enzyme differs in humans.

Previous studies in B16-F10 melanoma cells have shown that microphthalmia-associated transcription factor (MITF) expression has a strong correlation with the phenotypes of melanogenic enzymes, thereby contributing to melanin production. Upon stimulation by UV irradiation, intracellular α-melanocyte-stimulating hormone (α-MSH) is produced and then binds to melanocortin receptor 1 (MC1R) to activate adenylylated acid cyclase (AC) that in turn increases cyclic adenosine monophosphate (cAMP) levels. MITF expression is augmented by the increased phosphorylation of cAMP response element-binding protein (CREB) and protein kinase A (PKA). Recent studies revealed that the three serine/threonine kinases of mitogen-activated protein kinases (MAPks) such as p38 MAPK, extracellular signal-regulated kinase (ERK), and C-Jun N-terminal kinase (JNK), are involved in melanin production by regulating MITF expression. Specifically, ERK ubiquitination and phosphorylation induces the degradation of MITF, which then attenuates the expression of melanogenesis enzymes (tyrosinase, TRP1, and TRP2). Furthermore, ERK is known to negatively modulate the expression of MITF, indicating that several signals, including phosphatidylinositol 3-kinase (PI3 K)/protein kinase B (AKT) are associated with melanogenesis by regulating MITF expression.

Tyrosinase is an enzyme that limits the melanogenesis rate. The activator of tyrosinase is used as a tanning agent and in haircare products. However, since hair coloring agents can lead to adverse side effects, identifying nontoxic melanogenic agents has become a point of interest for the development of new functional cosmetics and treatments. Coumarin and derivatives belong to a group of colorless crystalline oxygen heterocyclic compounds, forming a methyl ester bond in the derivative 7,8-dimethoxy-4-methylcoumarin (DMMC). We then investigated the effects of the synthesized DMMC on various melanin-producing activities in B16-F10 melanoma cells.

Results

Methylation of DHMC

As described below, DHMC was subject to methylation process. The obtained methylated reaction product was purified using preparative high-performance liquid chromatography (HPLC), and its yield is over about 90% (Figure 1a). In addition, as shown in the mass spectrum in Figure 1(b), the tallest peak at m/z 193 was identified as that of ionized DHMC, and it showed that an [M + H]+ peak at m/z 221 was that of DMMC.

1H nuclear magnetic resonance (NMR) analyses further confirmed that the methylated product was DMMC, based on comparison with NMR data from a previous study in which DMMC was synthesized with the same approach. Specifically, DMMC was synthesized with the same approach. As described below, DHMC was subject to methylation process. The obtained methylated reaction product was purified using preparative high-performance liquid chromatography (HPLC), and its yield is over about 90% (Figure 1a). In addition, as shown in the mass spectrum in Figure 1(b), the tallest peak at m/z 193 was identified as that of ionized DHMC, and it showed that an [M + H]+ peak at m/z 221 was that of DMMC.

Effects of DHMC and DMMC on the Viability of B16-F10 Melanoma Cells

The level of cell viabilities relative to non-treated controls was measured and compared using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay and from which the appropriate treatment concentrations of DHMC and DMMC with no significant cytotoxicity were chosen. The cell viability was 78%, 38%, and 36% in the concentration of 50, 100, and 200 μM DHMC, respectively, compared with that of the corresponding controls (Dulbecco’s modified eagle medium [DMEM]); 0.2 μM α-MSH was used as a positive control. By contrast, DMMC was nontoxic to B16-F10 cells at these concentrations with no decrease in viability (Figure 2). These results demonstrated that changes in methylation contributed to improving the safety of DHMC with no cytotoxicity.

Comparison of Effects of DHMC and DMMC on Melanin Production

In B16-F10 melanoma cells without α-MSH treatment, DHMC did not impact the synthesis of melanin due to its cytotoxicity, whereas DMMC increased melanin production over the increasing order of DMMC concentration (Figure 3). Based on this result, we further focused on the synthesized DMMC that caused the most significant change in melanin production.
Effects of DHMC and DMMC on Tyrosinase Activity

Increased tyrosinase expression and activities are an indicator for the increased production of melanin. The activity of tyrosinase was enhanced by 242.8%, 345.1%, and 442.8% after DMMC treatment at 50, 100, and 200 μM, respectively, relative to the untreated control (Figure 4). These results indicated that DMMC treatment enhances melanogenesis in accompany by the increase of tyrosinase activity in B16-F10 melanoma cells. This posed DMMC as a potential dyeing agent.

DMMC Induces the Upregulation of Melanogenic Enzymes and MITF

The transcription factor MITF regulates melanogenic enzymes involved in melanogenesis, such as tyrosinase, TRP1, and TRP2. Relative to that of the untreated cells, MITF protein expression was increased in the increasing order of DMMC concentration at 48 h post-DMMC (Figure 5a). Similarly, the expression levels of other melanogenic proteins were increased after DMMC treatment for 48 h in a concentration-dependent manner relative to untreated cells (Figure 5b to d). These data suggest that DMMC treatment upregulated TRP1, TRP2, and tyrosinase production by increasing MITF expression.

DMMC Influences the AKT Signaling Pathway in B16F10 Melanoma Cells

We next focused on its effects on molecules in the AKT signaling pathway, which has been reported to regulate the

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**Figure 1.** (a) HPLC chromatogram of 7,8-dihydroxy-4-methylcoumarin (DHMC) and 7,8-dimethoxy-4-methylcoumarin (DMMC). (b) Structure and mass analysis of DHMC and DMMC. Abbreviation: HPLC: high-performance liquid chromatography.

**Figure 2.** Effect of DHMC and DMMC on cell viability determined by the MTT assay. Cells were treated with different concentrations (50, 100, and 200 μM) of DHMC and DMMC for 72 h. The values represent the mean ± SD of three repeated independent experiments performed. Significant difference from the untreated group: *P < .05, **P < .01, ***P < .005. Abbreviations: DHMC: 7,8-dihydroxy-4-methylcoumarin; DMMC: 7,8-dimethoxy-4-methylcoumarin.
Western blotting showed a reduction of AKT phosphorylation after DMMC treatment to melanoma cells in a concentration-dependent manner and its maximum reduction was achieved at 200 μM (Figure 6). This result indicated that DMMC distinctly enhances melanogenesis by significantly reducing AKT phosphorylation.

Figure 3. Effects of DHMC and DMMC on melanin production in B16-F10 melanoma cells treated with or without α-MSH (0.2 μM; positive control). Cells were treated with different concentrations (50, 100, and 200 μM) of DHMC and DMMC for 72 h. The values represent the mean ± SD of three repeated independent experiments performed. 
Note: Significant difference from the untreated group: *P < .05, **P < .01, ***P < .005.
Abbreviations: DHMC: 7,8-dihydroxy-4-methylcoumarin; DMMC: 7,8-dimethoxy-4-methylcoumarin; α-MSH: α-melanocyte-stimulating hormone.

Figure 4. Effects of DHMC and DMMC on intercellular tyrosinase activity in B16-F10 melanoma cells treated with or without α-MSH (positive control). The cells were treated with different concentrations (50, 100, and 200 μM) of DHMC and DMMC for 72 h. The values represent the mean ± SD of three repeated independent experiments performed.
Significant difference from the untreated group: ***P < .005.
Abbreviations: DHMC: 7,8-dihydroxy-4-methylcoumarin; DMMC: 7,8-dimethoxy-4-methylcoumarin; α-MSH: α-melanocyte-stimulating hormone.
DMMC Influences the MAPK Pathway in B16F10 Melanoma Cells

Western blotting showed that JNK protein expression levels increased with the increase in DMMC concentration (Figure 7a). However, DMMC decreased the expression level of ERK, and p38 (Figure 7b and c).

Discussion

The skin plays a key role in body protection against potentially harmful external stimulations. Melanin creates a barrier, for example, against the penetration of UV rays, and determines the color of the skin, hair, and eyes. In this study, we allowed DHMC to undergo methylation process and obtained the black compound DMMC. The structure was then confirmed by HPLC, ESI-MS, and NMR analyses. In contrast to DHMC, DMMC exhibited significantly enhanced melanin production in B16-F10 melanoma cells without significant cytotoxicity. The increased melanin content was accompanied by the increase in DMMC concentration, and also increased the activity and/or protein expression level of the melanogenesis enzymes TRP1, TRP2, and tyrosinase, and MITF a master regulator in melanogenesis. Based on previous studies, AKT signaling pathway was shown to play a role in melanin production in B16-F10 cells, and reduced AKT phosphorylation increases MITF expression by inducing CREB phosphorylation. Therefore, our results indicate that DMMC increases...
MITF expression through inhibition of AKT phosphorylation, thus inducing melanogenesis. Specifically, these results suggest that DMMC impacts the MAPK signaling pathway mainly and increased JNK phosphorylation may bind to the promoter region of MITF gene and boost MITF gene expression, in turn increasing the protein expression of tyrosinase, TRP1, and TRP2, and ultimately inducing melanin production.

Overall, DHMC showed cytotoxicity, whereas DMMC did not show cytotoxicity and increased melanin production. In this study, DMMC-induced melanogenesis occurred through increased phosphorylation of JNK, ERK, and p38, while decreasing AKT phosphorylation. Therefore, the results of this study suggest the potential use of DMMC as a tanning or pigmentation agent in cosmetics. However, animal studies need to be followed to ensure the safety and assess any arising side effects.

Materials and Methods

Methylation of DHMC

All chemicals were purchased from Sigma-Aldrich (St. Louis, MA, USA). In a typical methylation experiment, DHMC (50 mg) in dimethyl carbonate (4 mL) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.6 mmol, 10 mL) was heated at 110 °C overnight with magnetic stirring. Next, methanol (3 mL) was added and completely mixed by shaking. The mixture was then subject to evaporation. The resulting residues were dissolved in 1N HCL (5 mL) and ethyl acetate (10 mL) solvents. Finally, the ethyl acetate layer was vacuum-dried for 2 h at 40 °C.

HPLC Analysis and Purification of DMMC

Using Shimadzu SpectroMonitor 3200 digital UV-Vis detector coupled with a Shim-pack GIS C18 column (250 × 4.5 mm) (Shimadzu, Kyoto, JP), HPLC chromatograms were analyzed. The two degassed mobile phases are double distilled water supplemented with 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B). The overall percentage of solvent B was gradually changed from 10% to 100% for 30 min with a flow rate of 1 mL/min.

Liquid Chromatography–Mass Spectrometry (LC-MS) and NMR Analysis of DMMC

The LC system, Perkin-Elmer API (Norwalk, CT, USA) single-quadrupole MS, composed of an 1100 HPLC pump and 1100 HPLC Agilent Autosampler (Agilent Technologies) with an 1100 HPLC Agilent DAD Detector (220 to 500 nm). A reversed-phase column (Acquity BEH C18 2.1 mm × 100 mm [1.7 μm]) was used to separate the samples. ESI/MS detection of the samples: positive ion mode ESI+, acquisition range: 50-1, m/z 400, capillary voltage: 2.5 kV, cone voltage: 30 V, source temperature: 120°C, desolvation gas temp: 600°C, cone gas flow: 20 L h⁻¹, desolvation gas flow: 800 L h⁻¹. Analysis software MassLynx version 4.1 [Waters Corp.]. NMR spectra were generated using a VNMRS 500 NMR spectrometer [Agilent Technology] with a comparison of the residual solvent peak [DMSO-d₆, 500 MHz] of the reference peak from deuterated NMR solvents.

Cell Culture and Viability Assay

B16-F10 melanoma cells get from ATCC (American Type Culture Collection, Manassas, VA, USA) were cultured in phenol red-free DMEM supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS, Welande, Flaw Medium™, Gyeongsung, Korea) and 1% v/v penicillin and streptomycin (Welande, Fresh Media™, Gyeongsung, Korea) at 5% CO₂, 37 °C. The cells (1.0 × 10⁴ cells/well) were seeded into 24-well plates and incubated 24 h. Three different concentrations of DHMC and DMMC (50, 100, and 200 µM) or α-MSH (0.2 µM) were applied to the cells and incubated for 72 h. To assess cell viabilities, 1 mg/mL MTT reagent (VWR Life Science) was added to each well of the plates and incubated for 3 h. After the complete removal of culture medium and the remaining formazan crystals were solubilized in dimethyl sulfoxide (DMSO). Absorbance was read at 570 nm on a microplate reader (Thermo Fisher Scientific).

Measurement of Melanin Content

B16-F10 melanoma cells were seeded (4.0 × 10⁴ cells per well in 6-well plates) and cultured for 24 h. DHMC and DMMC (50, 100, and 200 µM) were added at different
concentrations in each well and cultured for 72 h with α-MSH (0.2 µM) as the positive control. To quantify intracellular melanin content, the spent medium was completely aspirated off followed by washing with phosphate-buffered saline. The resulting cell sediments were lysed in 1 mL of 1 M NaOH (added with 10% DMSO) at 90 °C for 1 h. Finally, the absorbance at 405 nm was read as described above.

**Assessment of Intracellular Tyrosinase Activity**

B16-F10 melanoma cell culture and sample treatments were made as described above. After culture was completed, the cells were trypsinized and pelleted by centrifugation at 848 g, 3 min. The proteins were isolated from the cells using 1% protease inhibitor cocktail-added 1X RIPA cell lysis buffer (Biosesang). The protein extraction process was facilitated by vortexing six times every 10 min, and then spun down at 15,928 g at −4 °C for 30 min. Protein amounts present in the supernatants were quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The supernatant was mixed with 2X Laemmli sample buffer to prepare a sample for western blotting. After heating at 90 °C for 5 min, and 20 µL of the sample was loaded onto a 10% sodium dodecyl sulfate gel for electrophoresis (SDS-PAGE) to separate proteins according to their size. The SDS-PAGE was subject to 110 V for 2 h and electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF). The blotted PVDF membrane was then blocked with 5% skim milk in Tris-buffered saline with Tween (TBST) buffer with 1 h shaking. Washed 6 times for 10 minutes using TBST buffer. The membranes were incubated overnight at 4 °C with primary antibodies. After that, washed with TBST buffer six times 10 min each. Secondary Antibody incubation for 2 h with the anti-mouse IgG or anti-rabbit IgG (Cell Signaling, USA) antibodies. Target proteins were visualized using an enhanced chemiluminescence kit (Bio-Rad) and band intensities were quantified with the aid of ImageQuant 1.3 (LAS-4000 mini, Fujifilm, Tokyo, Japan).

**Western Blotting**

The western blotting procedures and primary and secondary antibodies used in this study were previously published.37 B16-F10 melanoma cells seeded and cultured for 72 h as described above. The cells were treated with three different concentrations of DMMC (50, 100, and 200 µM). The DMMC-induced melanin production was compared against α-MSH (0.2 µM) only-treated positive control. Proteins were extracted in RIPA buffer containing 1% protease inhibitors. The lysates were spun down at 15,928 g at −4 °C for 30 min. Protein amounts present in the supernatants were quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The supernatant was mixed with 2X Laemmli sample buffer to prepare a sample for western blotting. After heating at 90 °C for 5 min, and 20 µL of the sample was loaded onto a 10% sodium dodecyl sulfate gel for electrophoresis (SDS-PAGE) to separate proteins according to their size. The SDS-PAGE was subject to 110 V for 2 h and electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF). The blotted PVDF membrane was then blocked with 5% skim milk in Tris-buffered saline with Tween (TBST) buffer with 1 h shaking. Washed 6 times for 10 minutes using TBST buffer. The membranes were incubated overnight at 4 °C with primary antibodies. After that, washed with TBST buffer six times 10 min each. Secondary Antibody incubation for 2 h with the anti-mouse IgG or anti-rabbit IgG (Cell Signaling, USA) antibodies. Target proteins were visualized using an enhanced chemiluminescence kit (Bio-Rad) and band intensities were quantified with the aid of ImageQuant 1.3 (LAS-4000 mini, Fujifilm, Tokyo, Japan).

**Statistical Analysis**

All experiments were conducted three times, and the results were presented as the mean ± standard errors from triplicate experiments. The significant difference from the untreated group: *P < .05, **P < .01, ***P < .005.

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Declarations of Conflicts of Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval
In applicable for this article.

ORCID iDs
Min-Sung Kang https://orcid.org/0000-0002-3238-139X
Sung-Chan Jang https://orcid.org/0000-0003-0379-4080
Taejin Park https://orcid.org/0000-0001-6831-7053
Seung-Young Kim https://orcid.org/0000-0002-9018-3116

Statement of Human and Animal Rights
This study contains no human or animal subjects.

Statement of Informed Consent
Informed consent is not applicable.

Trial Registration
Not applicable, because this article does not contain any clinical trials.

Supplemental Material
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