

# Dual Inhibition of $\gamma$ -Oryzanol on Cellular Melanogenesis: Inhibition of Tyrosinase Activity and Reduction of Melanogenic Gene Expression by a Protein Kinase A-Dependent Mechanism

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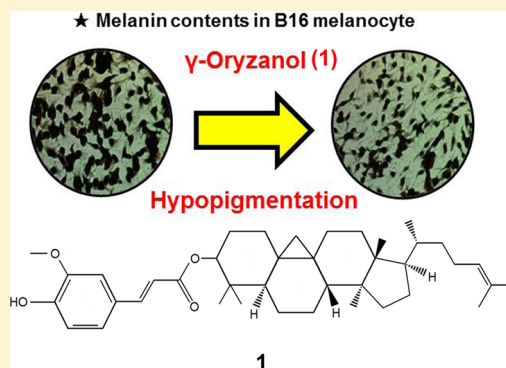
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**ABSTRACT:** The in vitro effects on melanogenesis of  $\gamma$ -oryzanol (**1**), a rice bran-derived phytosterol, were investigated. The melanin content in B16F1 cells was significantly and dose-dependently reduced (−13% and −28% at 3 and 30  $\mu$ M, respectively). Tyrosinase enzyme activity was inhibited by **1** both in a cell-free assay and when analyzed based on the measurement of cellular tyrosinase activity. Transcriptome analysis was performed to investigate the biological pathways altered by **1**, and it was found that gene expression involving protein kinase A (PKA) signaling was markedly altered. Subsequent analyses revealed that **1** stimulation in B16 cells reduced cytosolic cAMP concentrations, PKA activity (−13% for cAMP levels and −40% for PKA activity), and phosphorylation of the cAMP-response element binding protein (−57%), which, in turn, downregulated the expression of microphthalmia-associated transcription factor (MITF; −59% for mRNA and −64% for protein), a key melanogenic gene transcription factor. Accordingly, tyrosinase-related protein 1 (TRP-1; −69% for mRNA and −82% for protein) and dopachrome tautomerase (−51% for mRNA and −92% for protein) in **1**-stimulated B16F1 cells were also downregulated. These results suggest that **1** has dual inhibitory activities for cellular melanogenesis by inhibiting tyrosinase enzyme activity and reducing MITF and target genes in the PKA-dependent pathway.



Melanogenesis is regulated by various exogenous (e.g., ultraviolet light, X-rays, and photoaging chemicals) and endogenous (e.g., hormones and cytokines) signals and requires a complicated biosynthetic pathway that includes tyrosinase as a rate-limiting enzyme,<sup>1</sup> which catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and subsequent oxidation of DOPA to dopaquinone.<sup>2</sup> Hence, downregulation of tyrosinase activity has been proposed to be responsible for reduced melanin production.<sup>3</sup> Tyrosinase is modulated by microphthalmia-associated transcription factor (MITF), a master transcription factor in melanogenesis.<sup>4</sup> In addition, tyrosinase-related protein (TRP)-1 and dopachrome tautomerase are major targets of melanogenic enzymes induced by MITF.<sup>4</sup> Dopachrome tautomerase catalyzes the rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA),<sup>5</sup> whereas TRP-1 oxidizes DHICA to a carboxylated indole-quinone,<sup>6</sup> which is eventually converted into melanin. MITF activation is positively regulated by the cAMP-dependent protein kinase A (PKA) signaling pathway; thus, active PKA, following increased cellular cAMP levels, phosphorylates

cAMP-responsive element binding protein (CREB), and the transcription of MITF and subsequently its target genes are induced for melanin synthesis.<sup>4,7,8</sup>

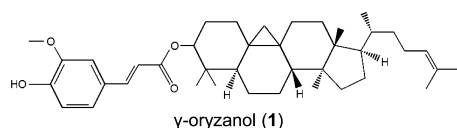
Traditional depigmenting agents such as hydroquinone, corticosteroids, and kojic acid remain highly effective. However, their use has been associated with several safety concerns regarding long-term exposure (e.g., atrophy, carcinogenesis, ochronosis, and other local or systemic side effects).<sup>9</sup> Thus, there is growing interest in identifying botanical and other natural extracts that have minimal side effects and result in significant hypopigmentation. Active compounds isolated from plants such as arbutin, aloesin, and certain flavonoids inhibit melanogenesis by different mechanisms without melanocytotoxicity.<sup>9</sup> Rice has also been suggested as a potential natural source of hypopigmentation agents.<sup>10</sup>

Rice (*Oryza sativa* L., Gramineae) bran has been used as a hypopigmenting agent in Korea, mainland China, and Japan for

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hundreds of years. Rice bran contains a variety of well-known antioxidative and bioactive compounds.<sup>11</sup> However, the key active hypopigmenting compound in rice bran is largely unknown except for ferulic acid, which shows a mild effect.<sup>12</sup>  $\gamma$ -Oryzanol (**1**) is a triterpene ester that is abundant in rice bran (0.2–0.6 mg/g of rice) and has several human bioactivities.<sup>13–15,16</sup> Eighteen single compounds from rice bran were previously isolated and screened for hypopigmenting activity. The results showed that **1** significantly reduced cellular melanin content; thus, its mechanism was investigated in cultured melanocytes. In the present study, the inhibitory effects of **1** on melanogenesis were examined. In addition, the mechanism underlying the observed hypopigmentation was investigated in B16F1 melanoma cells.

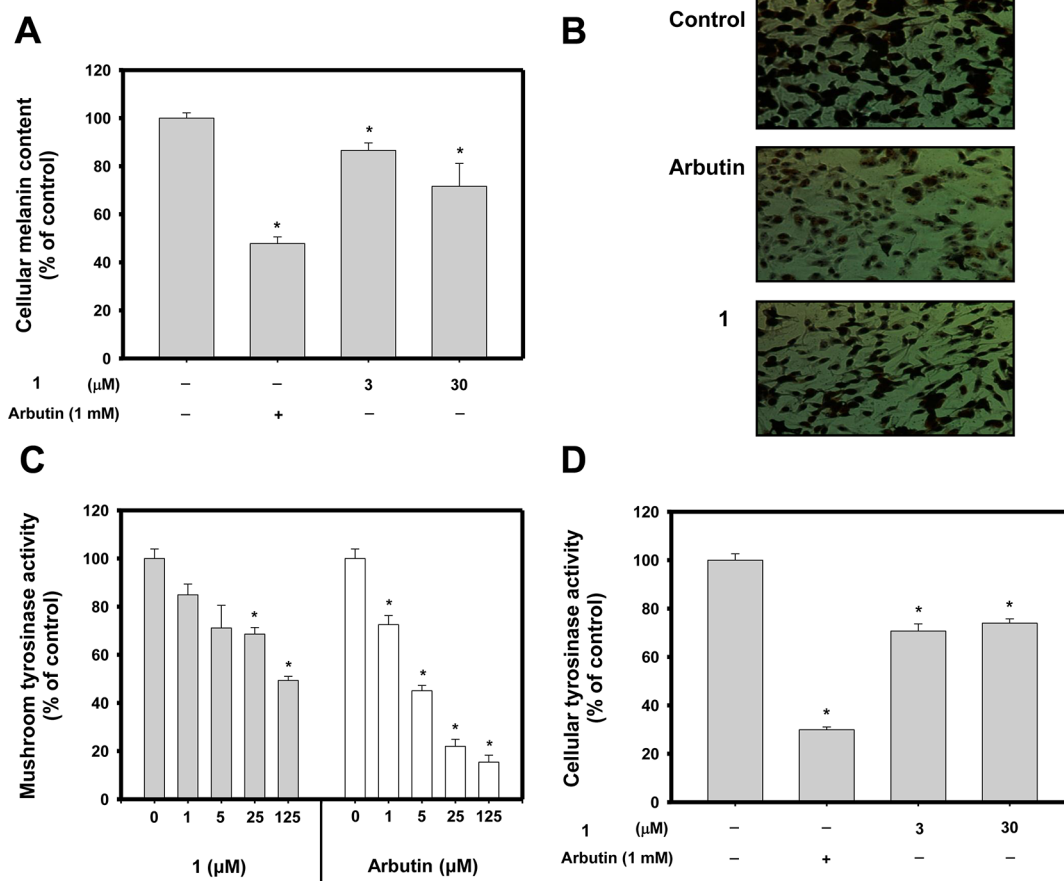


## RESULTS AND DISCUSSION

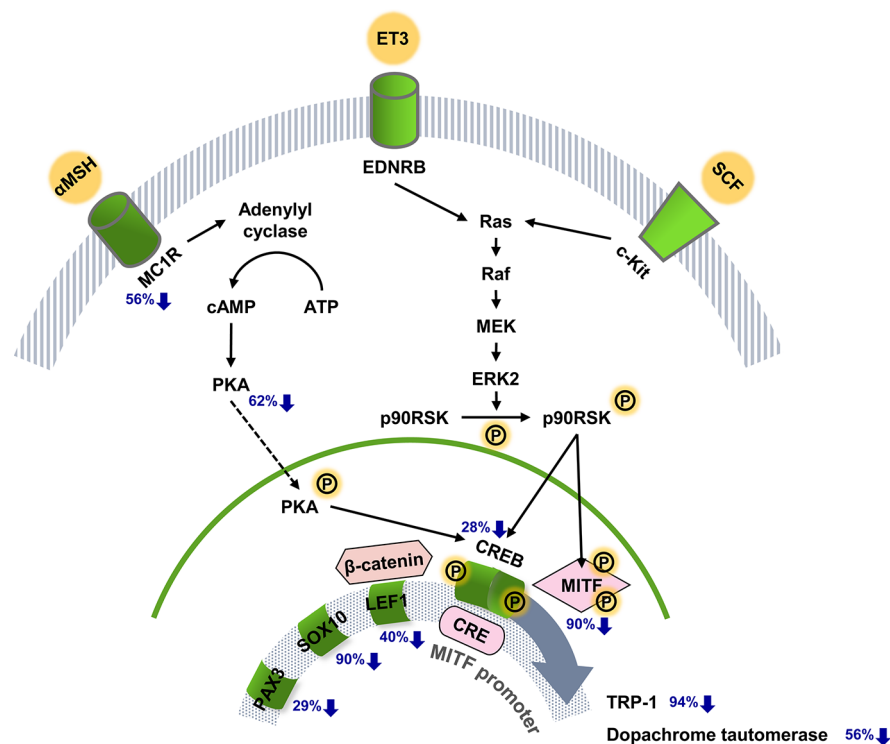
**Cellular Melanin Content.** The 18 single compounds that were isolated from rice bran were screened in a preliminary manner by measuring cellular melanin content in cultured

melanocytes. The data showed that **1** has a potent and significant inhibitory effect on melanogenesis; thus, the mechanism of **1** on hypopigmentation was examined further. Other studies have reported multiple bioactivities of **1** with no significant cytotoxicity in a cultured cell system, and this noncytotoxicity was confirmed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (concentrations of 300–0.03  $\mu$ M were tested). Cellular melanin content decreased significantly by 13% and 28% at 3 and 30  $\mu$ M **1**, respectively, compared to that in the controls (Figure 1A). Fontana–Masson staining showed a dramatic reduction in cytosolic melanin as a brown-black pigment in **1**-stimulated B16F1 melanoma cells compared to that in control cells (Figure 1B). Arbutin was used as the positive control in the experiment.<sup>17</sup>

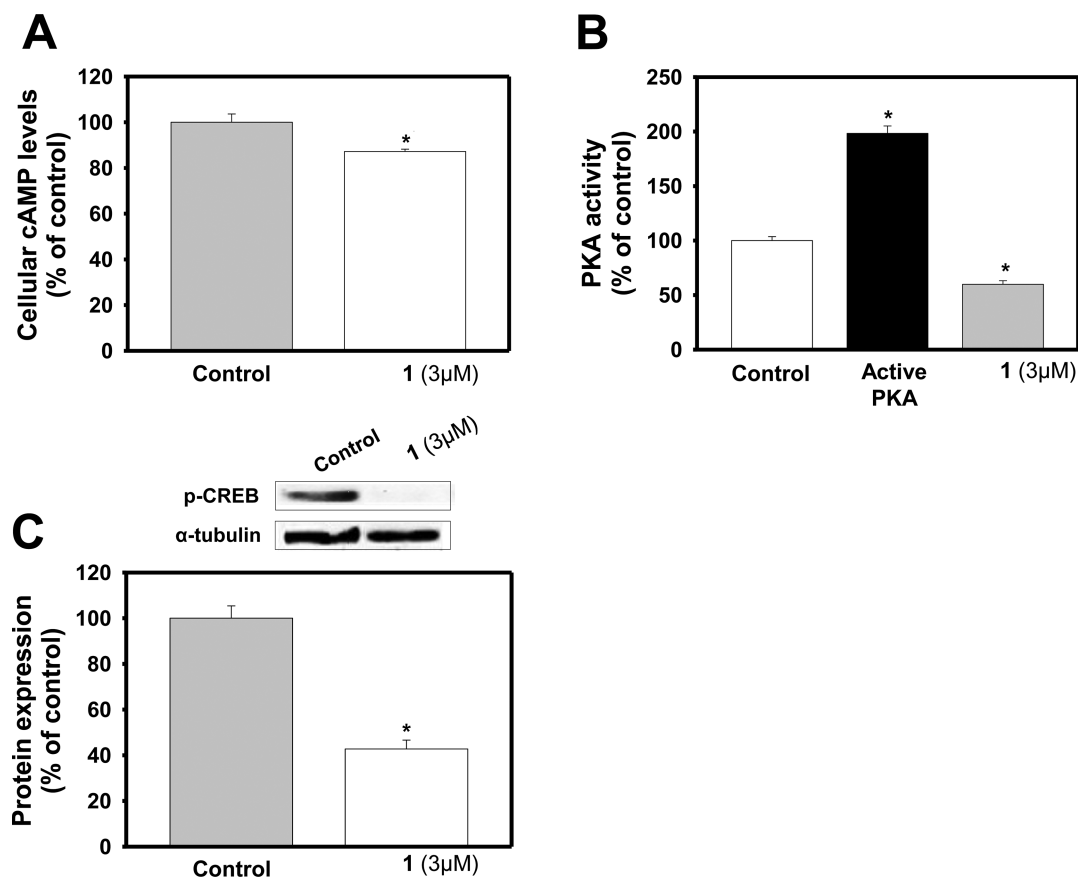
**Tyrosinase Activity.** Hypopigmentation can be achieved either by inhibiting tyrosinase enzyme activity or by reducing melanogenic enzyme expression.<sup>3,4,6</sup> Compound **1** effectively reduced tyrosinase activity in a dose-dependent manner, but the effect was slightly weaker than that of arbutin (Figure 1C). Arbutin is cytotoxic, whereas **1** is nontoxic at 5 mM;<sup>17,18</sup> thus, cells were stimulated with a high concentration of **1**. The level of tyrosinase activity was also measured in B16F1 melanoma cells treated with 3 or 30  $\mu$ M **1** for 72 h. Stimulation of **1** significantly decreased cellular tyrosinase activity by 29% and 26% at 3 and 30  $\mu$ M, respectively (Figure 1D). The inhibitory



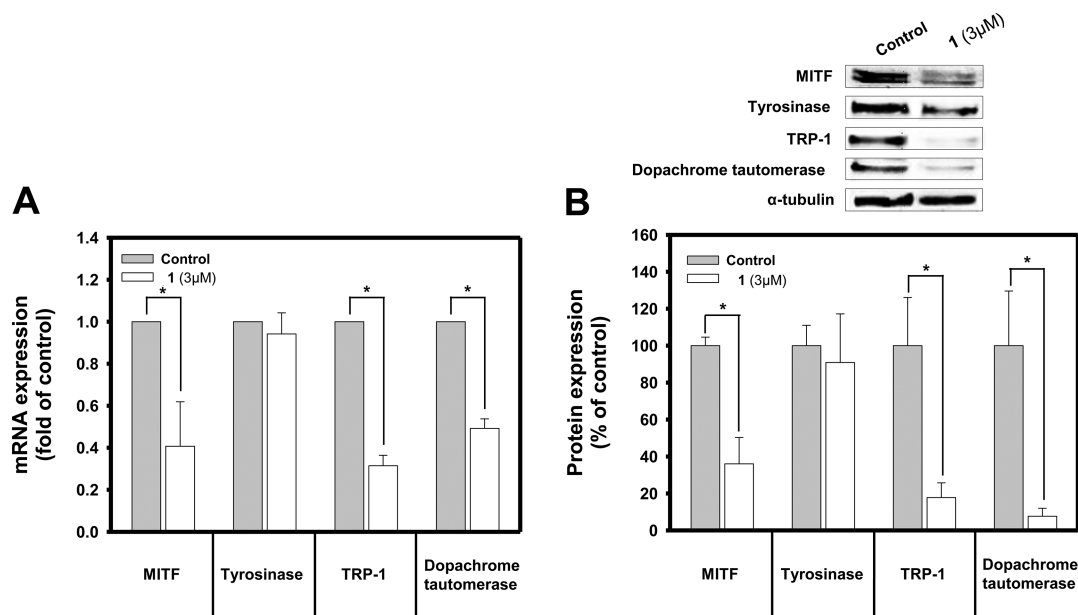
**Figure 1.** Effect of  $\gamma$ -oryzanol (**1**) on melanin synthesis and tyrosinase activity. (A) Cellular melanin content. B16F1 cells were treated with **1** (3, 30  $\mu$ M) or arbutin (positive control) for 72 h. (B) Melanin was stained brown using the Fontana–Masson staining method in cells stimulated with **1** (30  $\mu$ M). (C) Cell-free tyrosinase activity was measured using mushroom tyrosinase. (D) Cellular tyrosinase activity was determined using B16F1 cell lysates and **1** (1–125  $\mu$ M). B16F1 cells were treated with **1** (3, 30  $\mu$ M) or arbutin (positive control) for 72 h (\* $p$  < 0.05 compared to the control (no treatment); data are means  $\pm$  SE of three independent experiments).



**Figure 2.** Effects of **1** on the microphthalmia-associated transcription factor (MITF)-dependent melanogenic mechanism suggested by the oligonucleotide microarray analysis.



**Figure 3.** Effects of **1** on cAMP signaling. (A) Cellular cAMP levels in B16F1 cells treated with **1** for 72 h. (B) Protein kinase A (PKA) activity. (C) Phosphorylated CREB expression in **1**-treated B16F1 cells. Cells were stimulated with **1** (3  $\mu$ M) (\* $p$  < 0.05 compared to the control (no treatment); data are means  $\pm$  SE of three independent experiments).



**Figure 4.** Effects of **1** (3 μM) on microphthalmia-associated transcription factor (MITF) expression and its responsive genes (melanogenesis, tyrosinase, TRP-1, and dopachrome tautomerase) in B16F1 melanoma cells. B16F1 cells were treated with **1** for 72 h. (A) MITF, tyrosinase, tyrosinase-related protein (TRP)-1, and dopachrome tautomerase mRNA expression. Total RNA was extracted from B16F1 cells and analyzed for the expression of specific mRNAs by quantitative real-time PCR. (B) MITF, tyrosinase, TRP-1, and dopachrome tautomerase protein expression. Total protein was isolated from B16F1 cells and analyzed for the expression of specific proteins by immunoblotting (\**p* < 0.05 compared to the control (no treatment); data are means ± SE of three independent experiments).

effects of **1** on cellular tyrosinase activity at either concentration were not significantly different.

**Transcriptome Profiling.** Melanogenesis is a highly complicated and finely regulated biological process; thus, multiple mechanisms are present for hypopigmentation. Transcriptome profiling was conducted in **1**-stimulated melanocytes to identify the key antimelanogenic mechanism regulated by this compound. The data suggested that **1** downregulated the expression of PKA pathway genes; thus, MITF, the key transcription factor in melanogenesis and its target genes were downregulated (Figure 2). MITF expression decreased by 90%, TRP-1 by 94%, and dopachrome tautomerase by 56%, respectively, in **1**-treated (3 μM) B16F1 cells. In addition, alterations in multiple genes responsible for regulating the MITF promoter were detected in a microarray analysis. Compound **1** reduced the expression of paired-box 3, sex-determining region Y-box 10, and lymphoid enhancer factor by 29%, 90%, and 40%, respectively, all of which are involved in MITF transcription.<sup>19</sup> Reduced CREB expression (−28%) was also identified using microarray analysis. MITF transcription is also modified by receptor signaling pathways, including those of melanocortin-1 receptor (MC1R), endothelin receptor B, and c-kit.<sup>19</sup> In the presence of **1**, MC1R expression was suppressed by 56%, which may contribute to inhibition of cAMP signaling, reduction of cAMP-dependent PKA activity, and p-CREB and transcription of MITF sequentially, as assessed by targeted biomarker analysis.

**cAMP Concentration, PKA Activity, and MITF-Dependent Transcription.** The effect of **1** on PKA signaling and the expression of genes related to melanogenesis were investigated. Allosteric activation of PKA and subsequent CREB phosphorylation, particularly on serine 133, is a major stimulatory signal for MITF transcription. Active CREB (p-CREB) binds the cAMP-responsive element domain present in the MITF promoter,<sup>7</sup> and synthesized MITF participates in

self-induction as well as promoting melanogenesis target gene transcription.<sup>4</sup> Therefore, inhibiting PKA signaling and CREB phosphorylation may result in hypopigmented melanocytes; this was the case for **1**. Cells stimulated with **1** (3 μM) exhibited significantly suppressed cellular cAMP levels (−13%, Figure 3A), PKA activity (−40%, Figure 3B), and p-CREB expression (−57%, Figure 3C), which reduced the expression of MITF and its target genes TRP-1 and dopachrome tautomerase. In contrast, phosphorylation of extracellular signal-related kinase (ERK) 2 regulated by endothelin receptor type B (EDNRB) and c-kit induces MITF expression. However, ERK 2 gene expression and its phosphorylated form were unaltered (data not shown). These results suggest that the inhibitory effects of **1** on the MITF-dependent melanogenic mechanism were induced not by ERK2 stimulation, but by suppressing p-CREB during cAMP signaling (Figure 2).

**Expression of MITF and Its Responsive Genes TRP-1 and Dopachrome Tautomerase.** Next, the expression of selected genes and proteins in melanogenesis including MITF, a master regulator of melanogenesis,<sup>4</sup> and its target genes (tyrosinase, TRP-1, and dopachrome tautomerase) was examined. Compound **1** significantly reduced MITF, TRP-1, and dopachrome tautomerase mRNA expression by 59%, 69%, and 51%, respectively, compared to the control (Figure 4A). The protein expression pattern was similar to that of mRNA determined by quantitative real-time PCR. MITF, TRP-1, and dopachrome tautomerase protein levels decreased by 64%, 82%, and 92%, respectively (Figure 4B). However, tyrosinase expression was unaltered by stimulation using **1**.

Although the skin-whitening effects of rice bran are well-known, the mechanisms through which the compounds responsible for hypopigmentation exert their effects are largely unknown. In the present study, the results demonstrated that **1** is a key rice compound with a hypopigmentation effect.



Compound **1** directly inhibited tyrosinase enzyme activity and reduced the expression of melanogenic genes through a PKA-dependent signaling pathway. This study provides an understanding of the benefits of using natural resources and should promote the development of novel hypopigmentation products, from rice bran.

## ■ EXPERIMENTAL SECTION

**General Experimental Procedures.** B16 murine melanocytes were purchased from the Korean Cell Line Bank (Seoul, South Korea). Purified  $\gamma$ -oryzanol (**1**) was provided by the National Institute of Crop Science in Korea, and the purity was >95%, as determined by HPLC. Arbutin, mushroom tyrosinase, protease inhibitor cocktail, iQ SYBR Green Supermix, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA), whereas PowerScript reverse transcriptase was obtained from Clontech (Mountain View, CA, USA). Protein nitrocellulose transfer membranes were purchased from Whatman (Dassel, Germany). The ECL Western Blotting Analysis System was obtained from Amersham Biosciences (Piscataway, NJ, USA). The protein quantification reagent was obtained from Bio-Rad (Hercules, CA, USA), and the Fontana–Masson staining kit was purchased from American MasterTech Scientific, Inc. (Lodi, CA, USA). The cAMP enzyme immunometric assay and PKA kinase activity kits were purchased from Enzo Life Sciences (Farmingdale, NY, USA). RNeasy mini, RNase-Free DNase, and polymerase chain reaction (PCR) purification kits were purchased from Qiagen (Hilden, Germany). Cy3-dUTP and Cy5-dUTP were obtained from Amersham Biosciences. The Mouse OneArray chip was produced by the Phalanx Biotech Group (Hsinchu, Taiwan).

**Cell Culture.** B16F1 murine melanoma cells were purchased from the Korean Cell Line Bank (Seoul, South Korea) and cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin at 37 °C under 5% CO<sub>2</sub>.

**Cellular Melanin Content.** B16F1 melanoma cells cultured in six-well culture plates were treated with or without **1** for 72 h. After lysis and centrifugation at 12000g for 5 min at 4 °C, the cell pellets were dissolved in 1 N NaOH and incubated at 60 °C for 1 h. The absorbance was determined spectrophotometrically at a wavelength of 400 nm using synthetic melanin as a standard. The amount of cellular melanin was normalized to the total protein concentration (determined by the Bradford method).

**Fontana–Masson Staining.** B16F1 melanoma cells cultured on slides were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature and stained for melanin with a Fontana–Masson staining kit, according to the manufacturer's instructions. Briefly, the cells were stained with ammoniacal silver, gold chloride, and sodium thiosulfate.

**Cell-Free Tyrosinase Activity Assay.** Fifty microliters of 0.03% (w/v) tyrosine in distilled water, 75  $\mu$ L of 0.1 M phosphate buffer with or without **1** (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and 25  $\mu$ L of mushroom tyrosinase (400  $\mu$ L/mL) in 0.1 M phosphate buffer were added to each well of a 96-well microplate and incubated at 37 °C for 20 min. Absorbance was measured at a wavelength of 492 nm. Percentage tyrosinase inhibition was calculated as  $(1 - \text{sample OD}_{492} / \text{control OD}_{492}) \times 100$ .

**Cellular Tyrosinase Activity Assay.** B16F1 melanoma cells cultured in six-well culture plates were treated with or without **1** for 72 h. The cells were lysed with 1% (v/v) Triton-X/phosphate-buffered saline (pH 6.8) and centrifuged at 12000g for 5 min at 4 °C. Next, 70  $\mu$ L of supernatant was added to each well and mixed with 140  $\mu$ L of 0.1% (w/v) L-DOPA in a 96-well microplate. To show a linear increase in absorbance, the sample was measured at an absorbance wavelength of 475 nm during the first 2 h of the reaction. Tyrosinase activity was normalized to total protein concentration.

**Oligonucleotide Microarray Analysis.** Two-color oligonucleotide microarray experiments were performed with nontreated (control) and **1**-treated B16F1 melanoma cells. Total RNA was extracted from the cells using RNAiso Plus and further purified using

the RNase-free DNase I set (Qiagen) and the RNeasy MinElute Cleanup kit (Qiagen). cDNA was synthesized from 8  $\mu$ g of purified RNA using M-MLV reverse transcriptase, oligo(dT)20VN primers, and dNTPs, which were subsequently labeled with Cy3-dUTP and Cy5-dUTP (GeneChem Inc., Dajeon, South Korea). Labeled cDNA samples were purified using the QIAquick PCR purification kit (Qiagen) and then hybridized to the Mouse OneArray (Phalanx Biotech Group, Belmont, CA, USA), which contained 29 922 mouse genome probes. Hybridized arrays were scanned with the GenePix 4000B scanner (Axon Instruments, Sunnyvale, CA, USA), and the resulting images and probe-level gene expression values were visualized and computed by GenePix 5.1 software (Axon Instruments), respectively.

**Measurement of cAMP Levels.** Cellular cAMP levels in B16F1 melanoma cells treated with **1** for 72 h were quantified with a colorimetric competitive immunoassay kit from Enzo Life Sciences, according to the manufacturer's instructions.

**PKA Activity Assay.** PKA activity was determined using the solid-phase enzyme-linked immunosorbent assay kit from Enzo Life Sciences, according to the manufacturer's instructions.

**Quantitative Real-Time PCR Analysis.** Total RNA was extracted from B16F1 melanoma cells using Trizol reagent. Complementary (cDNA) was synthesized from 2  $\mu$ g of total RNA using M-MLV reverse transcriptase (Mbiotech, Inc., Seoul, Korea) and oligo(dT). Gene expression levels were measured using a Bio-Rad iQ5 iCycler system with RealMasterMix SYBR ROX (5 Prime, Hamburg, Germany). The primer sequences were as follows: MITF forward 5'-GTATGAACACGCACTCTCTC-3' and reverse 5'-CTTCTGCGCTCATACTGCTC-3'; tyrosinase forward 5'-GGCCAGCTT TCAGGCAGAGGT-3' and reverse 5'-TGGTGCTTCATGGGCAAA ATC-3'; TRP-1 forward 5'-GCTGCAGGAGCCTTCTTTCTC-3' and reverse 5'-AAGACGCTGCACTGCTGGTCT-3'; dopachrome tautomerase forward 5'-GGATGACCGTGAGCAATGGCC-3' and reverse 5'-CGTTGTGACCAATGGGTGCC-3'; and cyclophilin forward 5'-GGCCGATGACGAGCCC-3' and reverse 5'-TGTCTTTGGAACCTT-3'. The reaction conditions were 95 °C for 3 min followed by 50 cycles of 95 °C for 10 s, 56 °C for 15 s, and 72 °C for 20 s. A melting curve of 71 cycles, starting at 55 °C and increasing by 0.5 °C every 10 s, was performed to determine primer specificity. Expression levels were calculated by the relative method, which compares the target and cyclophilin genes, using iQ5 Optical System software (version 2; Bio-Rad).

**Immunoblot Analysis.** Total protein was prepared as described previously.<sup>20</sup> Protein concentrations were determined by the Bradford assay. Proteins (40  $\mu$ g) were separated by 10% SDS-PAGE, then transferred to and immobilized on a nitrocellulose membrane. After blocking with Tris-buffered saline containing 5% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20, the membrane was probed with anti-MITF, antityrosinase, anti-TRP-1, antidopachrome tautomerase, anti-p-CREB, or anti- $\alpha$ -tubulin antibodies, followed by incubation with anti-rabbit, anti-mouse, or anti-goat IgG-HRP secondary antibodies. The primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactive bands were visualized with chemiluminescence reagents using the ChemiDoc XRS Imaging System (Bio-Rad). The relative band densities were determined using Gel-Pro Analyzer 4.0 (Media Cybernetics, Silver Spring, MD, USA). The target protein levels in each sample were normalized to those of  $\alpha$ -tubulin (internal reference).

**Statistical Analysis.** All data are expressed as means  $\pm$  standard errors. Values were compared using Student's *t*-test (*p*-values <0.05 were considered statistically significant).

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### Notes

The authors declare no competing financial interest.

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