

Mechanisms of melanogenesis inhibition by 2,5-dimethyl-4-hydroxy-3(2H)-furanone

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Summary

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Accepted for publication

21 January 2007

Key words

cAMP, cAMP response element binding protein, melanin, microphthalmia-associated transcription factor, protein kinase A, tyrosinase

Conflicts of interest

None declared.

J.L. and E.J. contributed equally to this work.

Background Increased production and accumulation of melanin is characteristic of a large number of skin diseases, including acquired hyperpigmentation such as melasma, postinflammatory melanoderma and solar lentigo. Thus, there is an increasing need for the development of depigmenting agents.

Objectives To evaluate the depigmenting capacity of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) and to elucidate the mechanisms by which it inhibits α -melanocyte-stimulating hormone (α -MSH)-induced melanogenesis in B16 melanoma cells *in vitro*.

Methods Several experiments were performed in B16 melanoma cells. We studied melanin content, tyrosinase activity and cAMP production, and performed cAMP response element (CRE) luciferase reporter assay and Western blots for proteins involved in melanogenesis.

Results The melanin content and tyrosinase activity induced by α -MSH were inhibited significantly by DMHF. To clarify the mechanism of the depigmenting property of DMHF, we examined the involvement of DMHF in cAMP signalling induced by α -MSH. In CRE luciferase reporter assay, CRE reporter activation induced by α -MSH was inhibited by DMHF. Additionally, although DMHF did not inhibit cAMP production by α -MSH, both CRE binding protein (CREB) phosphorylation and the reduction of glycogen synthase kinase-3 β phosphorylation by α -MSH were blocked by DMHF. These data suggest that DMHF inhibits the downstream step of cAMP production induced by α -MSH, consequently inhibiting melanogenesis. This suggestion was further confirmed by the fact that the increased production levels of microphthalmia-associated transcription factor, tyrosinase and tyrosinase-related protein-1 induced by α -MSH were all reduced by DMHF in B16 melanoma cells.

Conclusions Our study shows that DMHF inhibits α -MSH-induced melanogenesis by suppressing CREB phosphorylation, which is induced by protein kinase A, and suggests that DMHF may be an effective inhibitor of hyperpigmentation.

Skin pigmentation, resulting from the production and distribution of melanin in the epidermis, is the major physiological defence against solar irradiation. In mammals, melanin synthesis is stimulated by a large number of effectors, including 1-oleyl-2-acetyl-glycerol,¹ ultraviolet (UV) B radiation,² cAMP-elevating agents [forskolin, 3-isobutyl-1-methylxanthine, α -melanocyte-stimulating hormone (α -MSH), glycyrrhizin]³⁻⁶ and placental total lipid fraction.⁷ Thus far, three major signalling pathways have been found to induce melanogenesis: one

of these is the protein kinase C-mediated pathway, and the second is the cAMP-mediated pathway. While the role of protein kinase C in the induction of melanogenesis remains controversial, compelling data have shown that the cAMP pathway plays a key role in the regulation of melanogenesis, augmenting the enzyme activity of pre-existing tyrosinase and increasing the amount of tyrosinase mRNA.³⁻⁵ Through the activation of protein kinase A (PKA) and cAMP response element (CRE) binding protein (CREB) transcription factors,

cAMP promotes an increase in the expression of microphthalmia-associated transcription factor (MITF),⁸ a melanocyte-specific transcription factor crucial for melanocyte development and differentiation.^{9,10} As a result, MITF binds to and activates the tyrosinase promoter, which leads to the stimulation of melanogenesis.^{11,12} Finally, p38 signalling was recently shown to be involved in melanogenesis.^{7,13}

Hyperpigmentation is a common and distressing problem caused by various inflammatory skin disorders such as eczema, allergic contact dermatitis and irritant contact dermatitis.^{14,15} Epidermal and dermal hyperpigmentation may depend on either increased numbers of melanocytes or melanogenic enzyme activities.¹⁶ Abnormal release of α -MSH, as well as UV radiation, chronic inflammation, and rubbing of the skin, is a triggering factor for these disorders.^{17,18} As a result of its localization in skin, acquired hyperpigmentation has psychosocial and cosmetic relevance, and many efforts have focused on screening for both recognized and putative depigmenting agents. Thus far, several compounds have been identified as depigmenting agents. Among these are arbutin, hydroquinone and kojic acid.¹⁹

In our preliminary studies to screen depigmenting agents, we found that 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) inhibits melanogenesis induced by α -MSH. Therefore, in this report, we aimed to demonstrate the depigmenting effect of DMHF and its mechanism of action.

Materials and methods

Materials

Minimum essential medium (MEM) and antibodies to human tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). H-89, α -MSH, and antibodies to β -actin and forskolin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Mouse monoclonal antibodies to MITF (21D1418) were obtained from Abcam Inc. (Cambridge, MA, U.S.A.). Antibodies to CREB, phospho-CREB (Ser133) and phosphoglycogen synthase kinase (GSK)-3 β (Ser9) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN, U.S.A.). Chemiluminescence kit was purchased from Amersham Pharmacia Biotech (Amersham, U.K.). AP-1-Luc, CRE-Luc and NF- κ B-Luc reporter plasmids were purchased from Stratagene (La Jolla, CA, U.S.A.).

Cell cultures

B16 melanoma cells were cultured in MEM with 10% fetal bovine serum and penicillin/streptomycin (100 IU/50 μ g mL⁻¹) in a humidified atmosphere containing 5% CO₂ in air at 37 °C. Primary cultures of normal human epidermal melanocytes (C-102-5C; Cascade Biologics, Inc., Portland, OR, U.S.A.) derived from neonatal foreskin were cultured in Medium 254 (M-254-500; Cascade Biologics) supplemented with

human melanocyte growth supplement (HMGS; S-002-5; Cascade Biologics). HMGS contains bovine pituitary extract, fibroblast growth factor, hydrocortisone, heparin and phorbol 12-myristate 13-acetate.

Melanin content assay

B16 melanoma cells, pretreated with the indicated concentrations of DMHF or 10 μ mol L⁻¹ H-89 for 1 h, were treated for 48 h with α -MSH (10 ng mL⁻¹) or forskolin (20 μ mol L⁻¹). The cells were then analysed by melanin content assay. Normal human melanocyte cells were plated on to 25-mm culture flasks at a density of 2.5×10^5 cells. After 1 day of culture, the medium was changed to fresh medium containing DMHF or DMHF plus α -MSH (10 ng mL⁻¹) every other day for a total of 7 days.

Melanin contents of cultured B16 melanoma cells and normal human melanocytes were measured according to the method of Oka *et al.*²⁰ with a slight modification. The colours of cell pellets were evaluated visually, and pellets were solubilized in boiling 1 mol L⁻¹ NaOH for 10 min. Spectrophotometric analysis of melanin content was performed at 400 nm absorbance. The entire experiment was performed in triplicate and results were confirmed by three independent experiments.

Tyrosinase activity assay

Tyrosinase activity was determined spectrophotometrically as described earlier by Mallick *et al.*²¹ This assay was based on a method described by Nakazawa *et al.*,²² using L-DOPA as the substrate. Briefly, B16 melanoma cells, pretreated with the indicated concentrations of DMHF or 10 μ mol L⁻¹ H-89 for 1 h, were treated for 48 h with α -MSH (10 ng mL⁻¹) or forskolin (20 μ mol L⁻¹). The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and extracted by sonication in 100 μ L buffer [PBS containing 1% Nonidet P-40, 0.01% sodium dodecyl sulphate (SDS), 100 μ mol L⁻¹ phenylmethylsulphonyl fluoride, 0.1 mol L⁻¹ Tris-HCl, pH 7.2, and 1 μ g mL⁻¹ aprotinin] and then centrifuged at 10 000 g for 10 min. The supernatants collected were used for enzyme assays and their protein content was estimated by the method of Lowry *et al.*²³ using bovine serum albumin as the standard. Samples of cell extract supernatant were incubated in duplicate for 1 h at 37 °C in 1 mL of 0.1 mol L⁻¹ sodium phosphate buffer (pH 7.4) containing 0.1% L-DOPA. The absorbance was then monitored at 450 nm in a Shimadzu spectrophotometer (Model: UV-2401 PC, UV-VIS; Shimadzu Corporation, Kyoto, Japan) and compared with purified mushroom tyrosinase. The standard curve was linear within the range of experimental values.

Cytotoxicity assay

The general viability of cultured cells was determined by the reduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium, monosodium salt

(WST-8; Dojindo Laboratories, Kumamoto, Japan) to a highly water-soluble formazan dye. This assay was performed after B16 melanoma cells, pretreated with the indicated concentrations of DMHF or $10 \mu\text{mol L}^{-1}$ H-89 for 1 h, were treated for 48 h with α -MSH (10 ng mL^{-1}) or forskolin ($20 \mu\text{mol L}^{-1}$) at 37°C in a 5% CO_2 atmosphere. Ten microlitres of WST-8 solution was added to each well. Cells were then incubated at 37°C for 3 h and the absorbance was measured at 450 nm using a spectrophotometer (PowerWave; BioTek Inc., Highland Park, VT, U.S.A.). The entire experiment was performed in triplicate and results were confirmed by three independent experiments.

Western blot analysis

B16 cells, pretreated with the indicated concentrations of DMHF for 1 h, were treated with α -MSH (10 ng mL^{-1}) for 15 min or for 2 h to evaluate the level of phosphorylated CREB or phosphorylated GSK-3 β , respectively. Further, in order to measure the level of MITF, tyrosinase, TRP-1 and TRP-2, B16 cells, pretreated with the indicated concentrations of DMHF for 1 h, were treated for 48 h with α -MSH (10 ng mL^{-1}). B16 melanoma cell lysates were separated by SDS-polyacrylamide gel electrophoresis (16% acrylamide gels) and transferred to Hybond-C membranes. The blots were incubated for 1 h with tyrosinase (1 : 500 dilution), MITF (1 : 1000), β -actin (1 : 1000), CREB (1 : 1000), phospho-CREB (1 : 1000), phospho-GSK-3 β (1 : 1000), TRP-1 (1 : 1000) and TRP-2 (1 : 1000) antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The proteins were then visualized using the Amersham ECL system. Results were confirmed by three independent experiments.

Luciferase reporter assay

To assay for nuclear factor (NF)- κB , activator protein (AP)-1 and CRE promoter activities, B16 melanoma cells were transfected with NF- κB -Luc, AP-1-Luc or CRE-Luc reporters along with Renilla luciferase expression vector driven by thymidine kinase promoter (Promega, Madison, WI, U.S.A.) using SuperfectTM reagent (Invitrogen, San Diego, CA, U.S.A.). After incubation for 24 h, B16 melanoma cells, pretreated with the indicated concentrations of DMHF or $10 \mu\text{mol L}^{-1}$ H-89 for 1 h, were treated for 14 h with α -MSH (10 ng mL^{-1}) or forskolin ($20 \mu\text{mol L}^{-1}$). The cells were then harvested and lysed. Supernatants were assayed for luciferase activity. Luciferase activity was determined using a Dual Luciferase Assay system (Promega) and a LB953 luminometer (Berthold, Bad Wildbad, Germany); the activity was expressed as a ratio of the NF- κB -, AP-1- or CRE-dependent firefly luciferase activity to the control thymidine kinase Renilla luciferase activity (% control). Results were confirmed by three independent transfections.

cAMP immunoassay

B16 melanoma cells, pretreated with the indicated concentrations of DMHF or $10 \mu\text{mol L}^{-1}$ H-89 for 1 h, were

treated for 14 h with α -MSH (10 ng mL^{-1}) or forskolin ($20 \mu\text{mol L}^{-1}$). After that, the cAMP level was measured using a cAMP kit from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). In brief, B16 melanoma cells (7×10^4) were lysed in 0.1 mol L^{-1} HCl to inhibit phosphodiesterase activity. Supernatants were collected, neutralized and diluted. Following neutralization and dilution, a fixed amount of cAMP conjugate (alkaline phosphatase-labelled cAMP) was added to compete with cAMP in the cell lysates for sites on a rabbit polyclonal antibody immobilized on a 96-well plate. After one washing to remove excess conjugated and unbound cell lysate cAMP, a substrate solution was added to the wells to determine the bound enzyme activity. Colour development was then stopped, and the absorbance was read at 405 nm. The intensity of the colour was inversely proportional to the concentration of cAMP in the cell lysates. All studies were repeated three times.

Statistical evaluation

Means \pm SEM were calculated; statistical analyses of results were performed using the t-test for independent samples. $P < 0.05$ was considered significant.

Results

2,5-Dimethyl-4-hydroxy-3(2H)-furanone inhibits α -melanocyte-stimulating hormone-induced melanogenesis in B16 melanoma cells

DMHF is a hydroxylated, methylated furanone (Fig. 1) and has been established to perform anticataract,²⁴ antioxidative²⁵ and anticarcinogenic functions.²⁶ More specifically, the antioxidant activity of DMHF led us to question the possibility that it may exert an inhibitory effect on melanogenesis, because it is well known that oxidation processes play important roles in melanin production. To investigate the depigmenting effect of DMHF, melanin content and tyrosinase activity assays were performed in B16 melanoma cells. As shown in Figure 2 (a, b), both melanin content and cellular tyrosinase activity induced by α -MSH were reduced by DMHF. However, DMHF itself did not induce a depigmenting effect. H-89 was employed as a positive control.

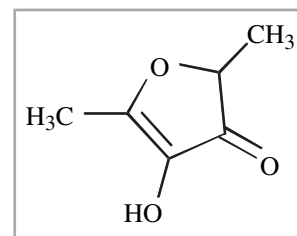


Fig 1. Structure of 2,5-dimethyl-4-hydroxy-3(2H)-furanone.

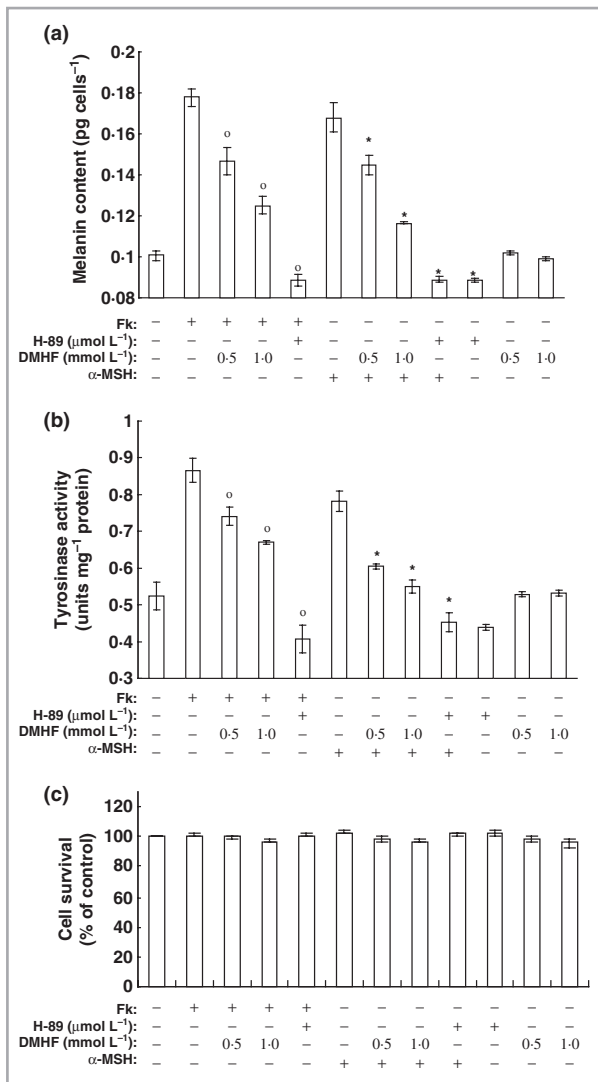


Fig 2. 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (DMHF) reduces both melanin content and tyrosinase activity induced by α -melanocyte-stimulating hormone (α -MSH) or forskolin (Fk). B16 cells, pretreated with the indicated concentrations of DMHF or 10 $\mu\text{mol L}^{-1}$ H-89 for 1 h, were treated for 48 h with α -MSH (10 ng mL⁻¹) or Fk (20 $\mu\text{mol L}^{-1}$). The cells were then analysed by melanin content and tyrosinase activity assays (a and b). Cell survival is shown (c). Data are expressed as mean \pm SEM. Results were confirmed by three independent experiments. * $P < 0.05$ vs. α -MSH, $^{\circ}P < 0.05$ vs. Fk.

However, it remained possible that the reduction of both melanin content and cellular tyrosinase activity may be induced by a cytotoxic effect of DMHF. To exclude this possibility, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in B16 melanoma cells. As shown in Figure 2c, we found that DMHF had no significant cytotoxic effect at any of the tested concentrations, demonstrating the depigmenting effect of DMHF.

Effect of 2,5-dimethyl-4-hydroxy-3(2H)-furanone on cAMP response element (CRE) promoter, CRE binding protein, and glycogen synthase kinase-3 β that are involved in melanogenesis

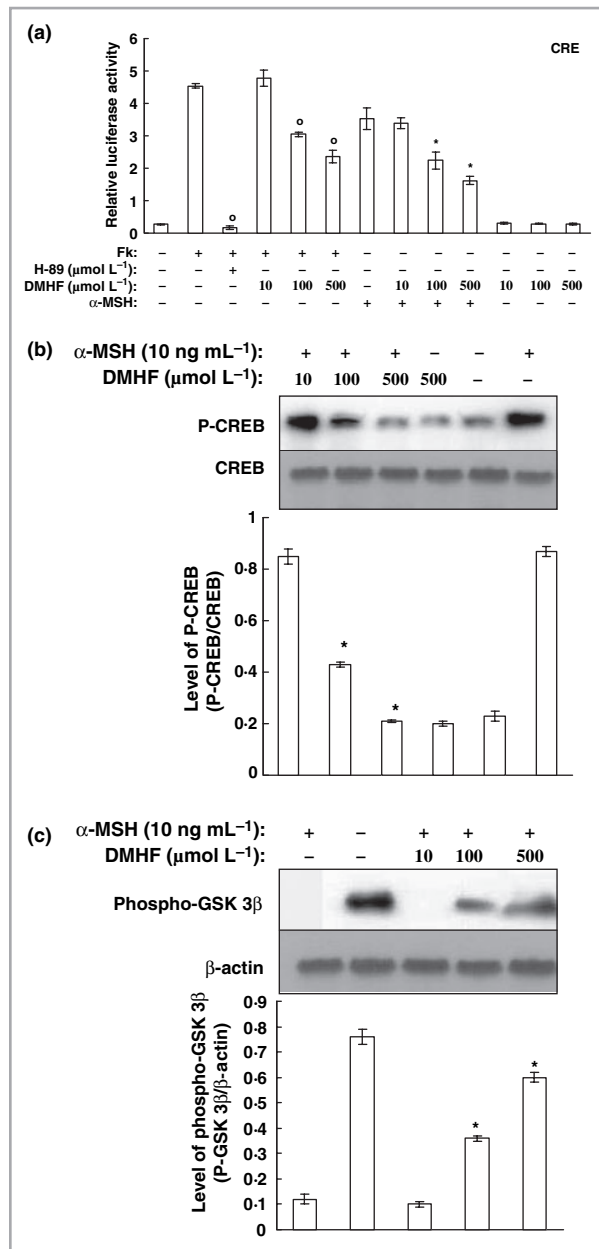
It has been reported that CRE, AP-1 and NF- κ B promoters are involved in melanogenesis.^{6,8,27} To determine the molecular mechanism of the depigmenting effect of DMHF, luciferase reporter assays were performed in B16 melanoma cells. As shown in Figure 3a, DMHF inhibited CRE luciferase reporter activation induced by α -MSH. However, we found that DMHF does not activate AP-1 or NF- κ B promoters, which are involved in the negative regulation of melanogenesis in B16 melanoma cells (data not shown). These findings suggest that DMHF may block melanogenesis through the suppression of cAMP signalling. To further demonstrate the detailed mechanism of action of DMHF in α -MSH-induced melanogenesis, we performed a cAMP production assay and a Western blot analysis for phosphorylated CREB and GSK-3 β . As shown in Figure 3b, DMHF did reduce α -MSH-induced CREB phosphorylation. Additionally, the decreased level of GSK-3 β phosphorylation induced by α -MSH was restored upon treatment with DMHF (Fig. 3c). However, cAMP production induced by α -MSH was not inhibited by DMHF (Fig. 4). These results suggest that DMHF operates downstream of cAMP production.

Effect of 2,5-dimethyl-4-hydroxy-3(2H)-furanone on the production of microphthalmia-associated transcription factor, tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 induced by α -melanocyte-stimulating hormone

It is well known that α -MSH induces tyrosinase production through the activation of MITF production. Therefore, we examined the effect of DMHF on the production of MITF and tyrosinase induced by α -MSH in B16 melanoma cells. As expected, DMHF inhibited production of both MITF and its downstream molecule, tyrosinase (Fig. 5). Additionally, because TRP-1 and TRP-2 are also known to play important roles in melanogenesis, we examined the effects of DMHF on TRP-1 and TRP-2. As shown in Figure 5, DMHF reduced the production of TRP-1, but not TRP-2.

2,5-Dimethyl-4-hydroxy-3(2H)-furanone inhibits α -melanocyte-stimulating hormone-induced melanogenesis in human primary melanocytes

As previously mentioned, we found that DMHF has a depigmenting effect in B16 melanoma cells. To investigate this depigmenting effect of DMHF in human primary melanocytes, a melanin content assay was performed. As shown in Figure 6, consistent with Figure 2, the increased melanin content induced by α -MSH was significantly reduced by DMHF, suggesting the possibility that DMHF might be introduced as a depigmenting agent for skin diseases such as melasma, post-inflammatory melanoderma and solar lentigo. A significant



cytotoxic effect of DMHF in human melanocytes was not observed (data not shown).

Discussion

In this study, we investigated the depigmenting effect and the molecular mechanism by which DMHF elicits its inhibitory effects on melanogenesis. We demonstrated that DMHF down-regulates tyrosinase expression, which strongly suggests that DMHF decreases melanogenesis through the inhibition of tyrosinase expression. Then, among the different pathways that are known to be involved in melanogenesis, including the AP-1, NF-κB and CRE pathway, we found that DMHF down-regulates tyrosinase expression via inhibition of the CRE activation pathway.

Fig 3. 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (DMHF) inhibits activation of cAMP response element (CRE) (a), phosphorylation of CRE binding protein (CREB) (b) and dephosphorylation of glycogen synthase kinase (GSK)-3β (c). (a) B16 melanoma cells were transfected with CRE-Luc reporter along with Renilla luciferase expression vector driven by thymidine kinase promoter (Promega, Madison, WI, U.S.A.) using Superfect™ reagent (Invitrogen, San Diego, CA, U.S.A.). After incubation for 24 h, B16 cells, pretreated with the indicated concentrations of DMHF or 10 μmol L⁻¹ H-89 for 1 h, were treated for 14 h with α-melanocyte-stimulating hormone (α-MSH) (10 ng mL⁻¹) or forskolin (Fk) (20 μmol L⁻¹). The cells were then harvested and assayed. Renilla luciferase vector was employed as a control for transfection efficiency, and the reporter data were processed using the dual luciferase method described in Materials and methods. Results were confirmed by three independent transfections. Data are expressed as luciferase activity relative to untreated control (mean ± SEM). *P < 0.05 vs. α-MSH, °P < 0.05 vs. Fk. (B, C) B16 melanoma cells, pretreated with the indicated concentrations of DMHF for 1 h, were treated for 15 min (b) or for 2 h (c) with α-MSH (10 ng mL⁻¹). Western blots were then performed with the phosphospecific antibodies phospho-CREB (P-CREB) (b) or phospho-GSK-3β (P-GSK 3β) (c). Detection of CREB (b) and β-actin (c) showed that each lane was loaded with equal amounts of protein. All studies were repeated three times. Results are shown as mean ± SEM. *P < 0.05 vs. α-MSH only.

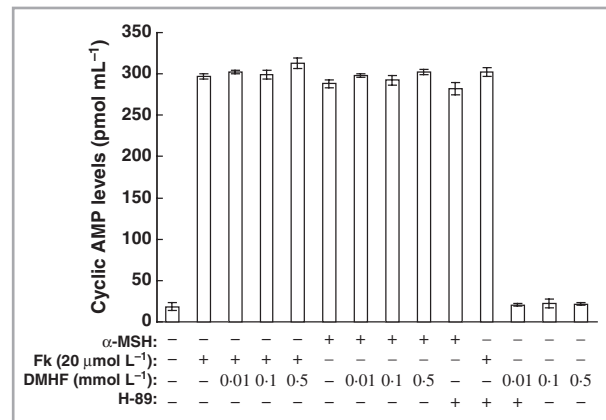


Fig 4. 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (DMHF) does not inhibit cAMP production. B16 melanoma cells, pretreated with the indicated concentrations of DMHF or 10 μmol L⁻¹ H-89 for 1 h, were treated for 14 h with α-melanocyte-stimulating hormone (α-MSH) (10 ng mL⁻¹) or forskolin (Fk) (20 μmol L⁻¹). After this, cAMP level was detected using a cAMP kit from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). All studies were repeated three times. Data are expressed as mean ± SEM.

Although DMHF has been established to perform anticarcinogenic functions, until now, the other functions of DMHF have not been investigated at all. However, as the biochemical synthesis of melanin is an oxidative process, the antioxidant activity of DMHF led us to investigate the possibility that it may exhibit a depigmenting effect. As shown in Figure 2, α-MSH-induced melanogenesis

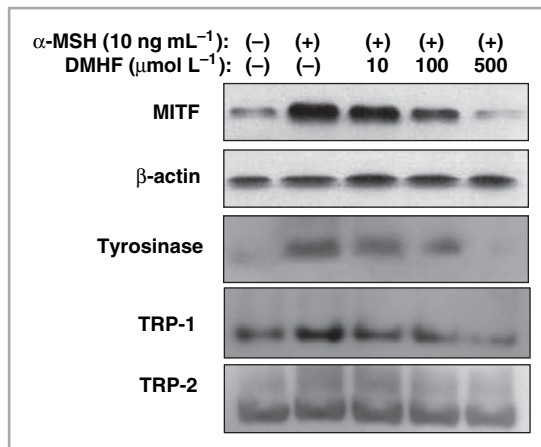


Fig 5. Effect of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) on α -melanocyte-stimulating hormone (α -MSH)-induced increase of microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 production. B16 cells that had been pretreated with or without DMHF for 1 h were stimulated with α -MSH (10 ng mL⁻¹) for 48 h. The cells were then subjected to Western blot analysis. Western blot was performed using antityrosinase antibody (Ab), anti-MITF Ab, anti-TRP-1 Ab, anti-TRP-2 Ab and anti- β -actin Ab in B16 melanoma cells.

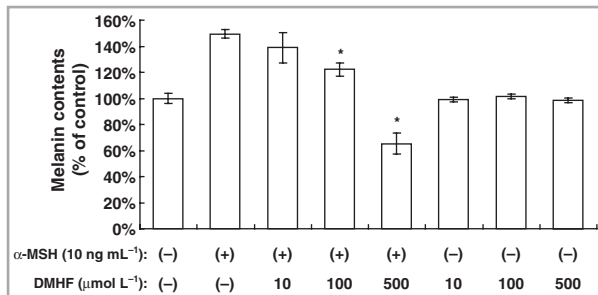


Fig 6. 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (DMHF) reduces melanin content induced by α -melanocyte-stimulating hormone (α -MSH) in human primary melanocytes. Human primary melanocytes, pretreated with the indicated concentrations of DMHF for 1 h, were treated with α -MSH (10 ng mL⁻¹). The medium was changed to fresh medium containing DMHF or DMHF plus α -MSH every other day for a total of 7 days. The cells were then analysed for melanin content. Data are expressed as mean \pm SEM. Results were confirmed by three independent experiments. * $P < 0.05$ vs. α -MSH.

was blocked by DMHF. To determine the mechanism of action of the depigmenting effect by DMHF, we performed AP-1, NF- κ B and CRE luciferase reporter assays. In our promoter assay we found that, while DMHF has no effect on the activation of AP-1 and NF- κ B promoters, the CRE promoter activation induced by α -MSH was blocked by DMHF. These findings suggest that the depigmenting effect of DMHF is mediated through inhibition of the CRE signalling pathway.

cAMP-induced melanogenesis has been reported to be mediated by a CRE promoter through the binding of CREB

family transcription factors that are phosphorylated and activated by PKA.²⁸ However, no canonical CRE was found in the mouse tyrosinase promoter. Thus, we hypothesized that DMHF might block melanogenesis through indirect inhibition of the tyrosinase promoter because MITF, which is known to be involved in melanogenesis by cAMP-elevating agents, has a CRE element in its promoter. As expected, like H-89 (a PKA inhibitor), DMHF reduced the melanin content induced by α -MSH, which indicates the involvement of the CRE pathway in DMHF-induced depigmentation. This result was further confirmed by the fact that DMHF reduced the phosphorylation and activation of CREB in B16 melanoma cells. However, cAMP production by α -MSH was not blocked by DMHF, which indicates that DMHF operates downstream of the cAMP production step. These properties of DMHF are very similar to those of piperlonguminine, which was recently reported to inhibit α -MSH-induced melanogenesis through the inhibition of CREB phosphorylation.²⁹ GSK-3 β has been widely implicated in cell homeostasis by its ability to phosphorylate a broad range of substrates, including glycogen synthase, the Tau microtubule-associated protein, and β -catenin.³⁰ A report recently showed that unphosphorylated GSK-3 β restores its activity and phosphorylates MITF on serine 289, thereby enhancing its binding to the tyrosinase promoter.³¹ Taking this report into consideration, we examined the possibility that GSK-3 β phosphorylation may be activated by DMHF. In our study, we found that GSK-3 β phosphorylation was activated by DMHF.

α -MSH is known to induce tyrosinase production through the activation of MITF production.³² In addition, TRP-1 and TRP-2 are the enzymes catalysing the major steps in synthesis of eumelanin.³³ Therefore, we examined the effect of DMHF on the production of MITF, tyrosinase, TRP-1 and TRP-2 induced by α -MSH in B16 melanoma cells. As expected, DMHF inhibited the production of both MITF and its downstream molecule, tyrosinase (Fig. 5). However, although DMHF reduced the production of TRP-1, TRP-2 production induced by α -MSH was not inhibited by DMHF. Recent experiments based on the use of antisense TRP-1 indicate that TRP-1 is required for the proliferation, morphology and tyrosinase activity of melanocytes and melanoma cells.³⁴ TRP-1 is also involved in tumour growth.³⁴ These results indicate that DMHF could be useful for the treatment of melanoma. However, as a specific TRP-1 inhibitor, a possible additional mechanism of depigmentation may be found in increased susceptibility to melanocyte cell death.

Taken together, the data gathered in this study demonstrate that DMHF inhibits melanogenesis by inhibiting the cAMP signalling pathway. Additionally, the fact that, while α -MSH-induced cAMP production was not blocked by DMHF, both the increased CREB phosphorylation and the decreased GSK-3 β phosphorylation induced by α -MSH were inhibited by DMHF, suggests that DMHF operates downstream of the cAMP production step. This means that DMHF inhibits melanogenesis by blocking CREB phosphorylation and GSK-3 β dephosphorylation by cAMP.

Acknowledgments

This work was supported by a grant from the Korean Ministry of Commerce, Industry, and Energy (C-9-1-10027152).

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