Received: 4 September 2020 | Revised: 14 December 2020 | Accepted: 5 January 2021

DOI: 10.1002/cbf.3620

RESEARCH ARTICLE

CELL BIOCHEMISTRY & FUNCTION WILEY

Diphlorethohydroxycarmalol inhibits melanogenesis via protein kinase A/cAMP response element-binding protein and extracellular signal-regulated kinase-mediated microphthalmia-associated transcription factor downregulation in α-melanocyte stimulating hormone-stimulated B16F10 melanoma cells and zebrafish

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Abstract

Diphlorethohydroxycarmalol (DPHC) is a marine polyphenolic compound derived from brown alga *Ishige okamurae*. A previously study has suggested that DPHC possesses strong mushroom tyrosinase inhibitory activity. However, the anti-melanogenesis effect of DPHC has not been reported at cellular level. The objective of the present study was to clarify the melanogenesis inhibitory effect of DPHC and its molecular mechanisms in murine melanoma cells (B16F10) and zebrafish model. DPHC significantly inhibited tyrosinase activity and melanin content dose-dependently in α-melanocyte stimulating hormone (α-MSH)-stimulated B16F10 cells. This polyphenolic compound also suppressed the expression of phosphorylation of cAMP response element-binding protein (CREB) by attenuating phosphorylation of cAMP-dependent protein kinase A, resulting in decreased MITF expression levels. Furthermore, DPHC downregulated MITF protein expression levels by promoting the phosphorylation of extracellular signal-regulated kinase. It also inhibited tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2 in α-MSH stimulated B16F10 cells. In in vivo studies using zebrafish, DPHC also markedly inhibited melanin synthesis in a dose-dependent manner. These results demonstrate that DPHC can effectively inhibit melanogenesis in melanoma cells in vitro and in zebrafish in vivo, suggesting that DPHC could be applied in fields of pharmaceutical and cosmeceuticals as a skin-whitening agent.

Significance of study: The present study showed for the first time that DPHC could inhibit a-MSH-stimulated melanogenesis via PKA/CREB and ERK pathway in melanoma cells. It also could inhibit pigmentation in vivo in a zebrafish model. This evidence suggests that DPHC has potential as a skin whitening agent. Taken together, DPHC could be considered as a novel anti-melanogenic agent to be applied in cosmetic, food, and medical industry.

KEYWORDS

anti-melanogenesis, diphlorethohydroxycarmalol, molecular mechanisms, zebrafish model

1 | INTRODUCTION

Melanin density correlates with skin darkness. Melanin is an essential component of skin to protect against ultraviolet radiation. Melanocytes are located in the bottom layer of the skin's epidermis. They are responsible for melanin synthesis.[1,2] In recent years, skin darkening is one of aesthetic problems of human beings. In particular, women prefer a fair skin to a dark skin. Skin-whitening products are becoming the most significant and continually growing segment in Asia skincare cosmetics market.[3] Besides preventing skin damage and complexion, numerous healthcare problems can be caused by excessive accumulation of melanin, such as pigmentation, petaloid actinic tanning, solar lentigo, and senile lentigines.[4] Hence, many kinds of whitening agents such as arbutin and kojic acid have been applied in the pharmaceutical and cosmetics market.[5,6] Natural whitening agents are securely used in cosmeceuticals so that safe and efficient natural whitening agents can be developed. They are essential subjects of cosmeceuticals research and development.

Melanin synthesis comprises a series of complex processes involving enzymatic and chemical reactions. Tyrosinase family proteins such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 play a critical role in melanin biosynthesis.[7,8] Down-regulation of tyrosinase, TRP-1, and TRP2 is essential for inhibiting melanin production.[8] Microphthalmia-associated transcription factor (MITF) is capable of monitoring transcription processes of melanogenic specific enzymes. It regulates the expressions of tyrosinase, TRP-1, and TRP-2.[9] Several signalling pathways play an essential role in melanogenesis by regulating MITF expression. MITF expression and activity are modulated by cAMP-dependent protein kinase A (PKA) and cAMP response element-binding protein (CREB). Phosphorylation of CREB is activated by phosphorylation of PKA. It stimulates the expression of MITF and results in an increase of melanin level.[10] Mitogen-activated protein kinase (MAPKs) family proteins including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) are also known to play an important role in melanogenesis.[11] Phosphorylation of p38 can induce MITF expression, whereas phosphorylation of ERK and JNK inhibits melanin synthesis by downregulating MITF expression and tyrosinase activity.[12]

Ishige okamurae, a brown alga, is widely distributed in the coast of Jeju Island in Korea. Diphlorethohydroxycarmalol (DPHC) is one of the most abundant bioactive compounds referred to as marine phlorotannins of *I. okamurae*. Previous studies have reported that DPHC possesses anti-obesity, anti-diabetic, and anti-oxidant effects.[13-15] Inhibitory effect of DPHC on melanogenesis and its protective effect against UV-B radiation-induced cell damage have also been reported.[16] These results indicate that DPHC possesses inhibitory effect against mushroom tyrosinase and suggest the potential of DPHC for suppressing melanogenesis.[16] However, the anti-melanogenesis activity of DPHC and molecular mechanisms involved in such activity at cellular level and in vivo have not been reported yet.

To further investigate the anti-melanogenesis potential and mechanism action of DPHC, the effect of DPHC on melanogenesis in a-MSH-stimulated B16F10 melanoma cells and in vivo using a zebrafish model was determined in the present study.

2 | MATERIALS AND METHODS

2.1 | Materials

Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (pH 7.4; PBS), foetal bovine serum (FBS) and penicillin-streptomycin (P/S) were obtained from Gibco BRL (Grand Island, New York). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-DOPA, arbutin, α-melanocyte stimulating hormone (α-MSH), and phenylmethanesulfonyl fluoride (PMSF) were purchased Sigma-Aldrich (St. Louis, Missouri). Tyrosinase, TRP1, TRP2 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, California). p-ERK, ERK, MITF, p-CREB, CREB, p-PKA and PKA were purchased from Cell Signalling Technology (Beverly, Massachusetts). Anti-rabbit IgG secondary antibody was purchased from Thermo Fisher Scientific (Waltham, Massachusetts).

2.2 | Extraction and isolation of DPHC

I. okamurae was collected from the coast of Jeju Island, Korea in April 2019. Protocols for extraction and isolation of DPHC were described in our previous study.[13] Briefly, *I. okamurae* was extracted with 70% ethanol to obtain an ethanol extract of *I. okamurae* (IO-E). IO-E was then partitioned with ethyl acetate to obtain an ethyl acetate fraction of IO-E (IO-E-EA). IO-E-EA was then subjected to silica gel and Sephadex-LH-20 column chromatography. DPHC (Figure 1A) was further purified by high-performance liquid chromatography.

2.3 | Cell culture

B16F10 mouse melanoma cells (KCLB 80008) were obtained from Korean Cell Line Bank (Seoul, Korea). Cells were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin. Cells were sub-cultured every 2 days and maintained at 37°C with 5% CO_2 in a humidified atmosphere incubator.

2.3.1 | Cell viability assessment

Cell viability was quantified through a colorimetric MTT assay[17] by measuring mitochondrial activity of viable cells. Briefly, B16F10 cells were seeded (5×10^4 cells/mL) into 96-well culture plates and incubated with various concentrations of samples for up to 72 hours prior to MTT treatment. An MTT stock solution (50 μL; 2 mg/mL in phosphate-buffered saline [PBS]) was added to each well to achieve a total reaction volume of 250 μL. After 4 hours of incubation, plates

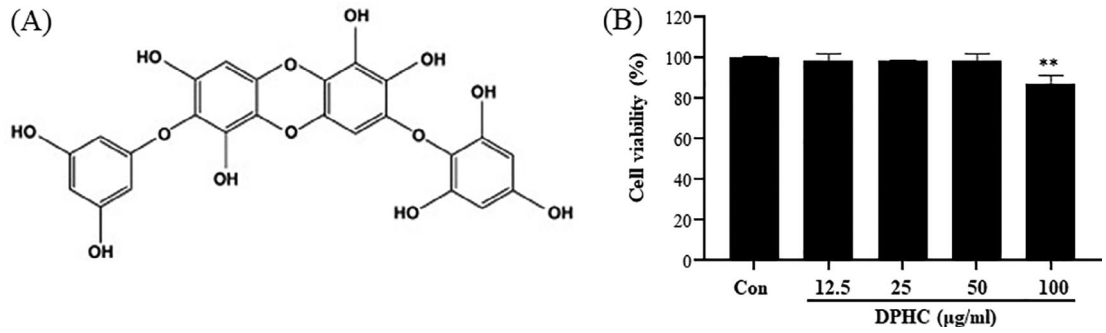

FIGURE 1 Chemical structure of DPHC A. Effect of DPHC on cell viability of murine melanoma treated for 72 hours B. Cell viability was measured using an MTT assay. Values are expressed as mean ± SD of triplicate experiments. **, $P < .01$ indicates a significant difference compared with the control group

were centrifuged at 2000 rpm for 10 minutes. After supernatants were aspirated, formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm using a spectrophotometer (Sunrise Tecan, Grödig, Austria).

2.3.2 | Assessment of melanin contents

Melanin contents were assessed using a published protocol[18] with slight modifications. Briefly, B16F10 cells (5×10^4 cells/mL) were seeded into 24-well plates and incubated in the presence or absence of 100 nM α-MSH. These cells were then incubated with various concentrations of samples for 72 hours. Plates were washed with PBS and then 1 N NaOH containing 10% DMSO was added into each well. After incubating at 80°C for 1 hour and mixing to solubilize melanin, the amount of melanin was assessed by measuring the absorbance at 475 nm.

2.3.3 | Assessment of tyrosinase activity

Tyrosinase activity was measured as previously described method[19] with minor modifications. Briefly, B16F10 cells were stimulated with α-MSH and treated with various concentrations of DPHC and arbutin. After incubation at 37°C for 72 hours, cells were washed with PBS and lysed in 50 nM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride (PMFS). Cell lysates were harvested by centrifugation at 12000 rpm for 30 minutes at 4°C. After quantification and normalization, cell lysate was incubated with 10 mM L-DOPA at 37°C for 1 hour for the formation of dopachrome. The solution was then measured using a spectrophotometer at 492 nm.

2.4 | Western blot analysis

After melanomas cells were treated with indicated concentrations of DPHC for indicated time, cells were washed with cold PBS twice and harvested. Next, cells were lysed with radioimmunoprecipitation assay buffer lysis buffer and centrifuged at 12000 rpm for 20 minutes at 4°C to harvest cell lysates. Protein concentrations were determined using a BCA protein assay kit. Samples containing equal protein contents were subjected to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation by electrophoresis, proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, California). Membranes were blocked with 5% non-fat dry milk in TBST (25 mM Tris-HCl, 137 mM NaCl, 2.65 mM KCl, 0.05% Tween 20, pH 7.4) for 2 hours at room temperature and then incubated with each of the following primary antibodies: MITF, tyrosinase, TRP1 and TRP2, p-CREB, CREB, p-PKA, PKA, p-ERK, ERK and GAPDH. Thereafter, membranes were washed with TBST and incubated with secondary antibodies for 2 hours at room temperature. Protein bands were visualized using an ECL western blotting detection kit (Santa Cruz, California) and captured with a FUSION SOLO Vilber Lourmat system (Paris, France).

2.5 | In vivo zebrafish assays

2.5.1 | Maintenance of parental zebrafish

Parental zebrafish were maintained as described previously.[20] Ten adult zebrafish were obtained from a commercial dealer (Seoul aquarium, Seoul, Korea) and kept in a 3 L acrylic tank at 28.5°C with a 14:10 hours light:dark cycle. These zebrafish were fed three times a day, 6 days/week, with tetramin flake food supplemented with live brine shrimps (*Artemia salina*; SEWHAPET food Co., Seoul, Korea). Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Embryos were collected within 30 minutes.

2.5.2 | DPHC treatment and determination of depigmentation effect in zebrafish embryos

Zebrafish embryos (n = 15) at approximately 7 to 9 hours post-fertilization (hpf) were transferred to individual wells of 24-well plates containing 450 μL embryo media. These embryos were treated with

designated concentrations of DPHC or arbutin and incubated. Evaluations of body pigmentation were carried out at 72 hpf. For observation, embryos were anaesthetised with 0.4% (w/v) tricaine and photographed under a microscope (Olympus, Japan).

Melanin contents of zebrafish embryos were determined as described previously.[21] Briefly, zebrafish embryos were treated with DPHC or arbutin and sonicated in Pro-Prep protein extraction solution (Intron Biotechnologies, Sungnam, South Korea). An untreated group of embryos served as a control sample. After centrifugation, the pellet was dissolved in 1 mL of 1 N NaOH at 100°C for 30 minutes. The mixture was then vigorously vortexed to solubilize the melanin pigment. Optical density of the supernatant was measured at 490 nm and the result was compared with the control sample, which was considered to represent 100% optical density. Melanin content was normalized to protein amount.

2.5.3 | Determination of UV-B-induced melanin contents in zebrafish embryos

UV-B-induced melanin contents in zebrafish embryos were determined as described previously.[22] According to the previously study, 50 mJ/cm^2 of UV-B could significantly increase melanin contents in zebrafish embryos. Therefore, 50 mJ/cm^2 of UV-B was applied to zebrafish embryos in the present study. At approximately 2 days post-fertilization (dpf), embryos (n = 15) were transferred to individual wells of a 24-well plate and maintained in embryo medium. Vehicle-only medium was used as control. Zebrafish embryos were incubated with indicated concentrations of DPHC or arbutin in zebrafish embryo medium for 1 hour. The incubation medium was removed and the zebrafish embryos were rinsed with fresh embryo medium. These zebrafish embryos were then layered in a glass slide, covered with sufficient embryo medium, and exposed to 50 mJ/cm^2 UVB (312 nm, UV lamp, BLX-LMC, Vilber Lourmat, France). All embryos were then incubated at 28.5°C in fresh medium for an additional 3 hours. Melanin contents of zebrafish embryos were determined as described in method Section 2.5.2.

2.6 | Statistical analysis

All experiments were carried out in triplicate. Data are described as mean ± SD. Statistical analysis was done using one-way analysis of variance (ANOVA) complemented by Duncan test's multiple range test. Statistical significance was considered at $P < .05$. Degrees of significance were indicated as follows: *, $P < .05$; **, $P < .01$; and ##, $P < .01$.

3 | RESULTS

3.1 | Cytotoxic effect of DPHC on melanoma cells

To evaluate whether DPHC was cytoxic, B16F10 cells were treated with DPHC at indicated concentrations for 72 hours. Cell viability was then measured via MTT assay. As shown in Figure 1B, DPHC did not exhibit cytotoxic effect on B16F10 cells at a concentration range of 12.5-50 μg/mL. Thus, DPHC concentrations of 12.5-50 μg/mL without showing cytotoxicity were applied in this study to further determine effects of DPHC on intracellular tyrosinase activity and melanin synthesis.

3.2 | Effects of DPHC on intracellular tyrosinase activity and melanin synthesis in α-MSH-stimulated melanoma cells

To verify the inhibitory effect of DPHC on α-MSH-mediated melanogenesis, we determined the quantity of intracellular melanin and tyrosinase activity in the presence of α-MSH. As shown in Figure 2A,

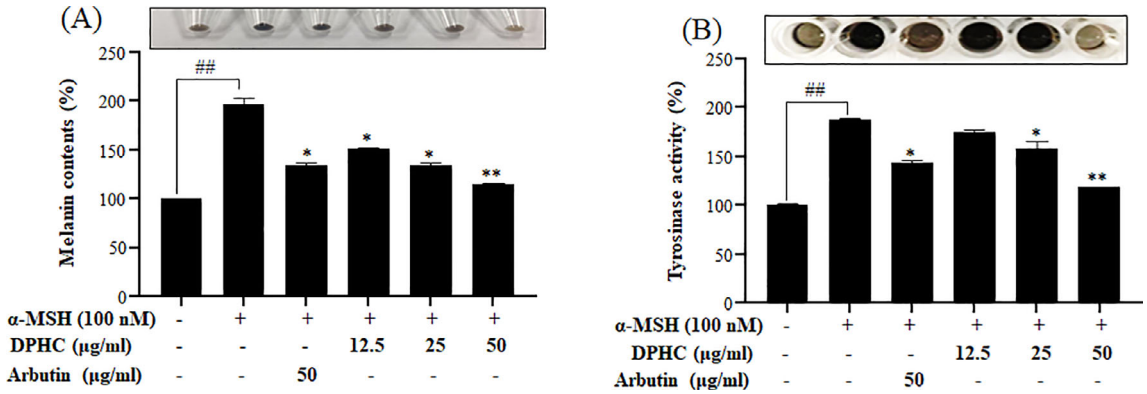

FIGURE 2 Effects of DPHC on melanin synthesis A, and intracellular tyrosinase activity B, in α-MSH-stimulated murine melanoma cells. Cells were exposed to 100 nM α-MSH in the presence of DPHC at indicated concentrations or 50 μg/mL arbutin. Percentage values of treated cells are expressed compared to that in control cells. Arbutin was used as a positive control. Values are expressed as mean ± SD of triplicate experiments. ##, $P < .01$ indicates a significant difference compared to the control group. *$P < .05$ and **$P < .01$ indicate a significant difference compared to only α-MSH-treated group

when B16F10 cells were treated with α-MSH, cellular melanin content was significantly increased in comparison with cells treated with control (absence of α-MSH). However, DPHC dose-dependently decreased the intracellular melanin content compared to cells treated with α-MSH alone. Tyrosinase is one of the key enzymes in mammalian melanin synthesis. We evaluated tyrosinase inhibition effects of DPHC to determine whether DPHC could directly affect intracellular tyrosinase activity. Our results showed that DPHC treatment reduced the increase of intracellular the tyrosinase activity induced by α-MSH in a dose-dependent manner (Figure 2B). Moreover, DPHC was more effective than arbutin (a positive control), a well-known melanogenesis inhibitor, at the same concentrations. These results suggest that DPHC could inhibit tyrosinase activity and that this inhibitory effect may lead to decreased cellular melanin synthesis in B16F10 cells.

3.3 | Effects of DPHC on tyrosinase, TRP-1, TRP-2, and MITF protein expression in α-MSH-stimulated melanoma cells

To elucidate the mechanisms underlying the anti-melanogenic effect of DPHC, we investigated whether DPHC could influence expression levels of melanogenesis-related proteins such as tyrosinase, TRP-1 and TRP-2 by Western blot. As shown in Figure 3, tyrosinase, TRP-1 and TRP-2 protein expression levels in α-MSH-stimulated B16F10 cells were reduced by treatment with DPHC in a dose-dependent manner compared to those in cells treated with α-MSH alone. MITF is a critical transcription factor that controls expression of melanogenic key proteins such as tyrosinase, TRP-1 and TRP-2. Therefore, we also investigated the expression of MITF protein after DPHC treatment. DPHC also dose-dependently inhibited MITF expression in α-MSH-stimulated B16F10 cells (Figure 3). These results suggest that inhibition of melanogenesis by DPHC is associated with the inhibition of tyrosinase activity and melanin synthesis through downregulation of MITF protein expression.

3.4 | Effects of DPHC on phosphorylation of ERK, PKA and CREB in α-MSH-stimulated melanoma cells

Previous reports have revealed that MITF expression is regulated by PKA, CREB and ERK signalling pathways in melanocytes. To further understand the mechanisms involved in the inhibition of melanogenesis by DPHC, we also determined whether DPHC could influence signalling pathways in α-MSH-stimulated B16F10 cells. Results showed that ERK phosphorylation was induced at 30 minutes after treatment with DPHC in α-MSH-stimulated B16F10 cells. ERK phosphorylation level was markedly increased at all indicated time points (Figure 4). Our results suggest that DPHC can induce the ERK signalling pathway in B16F10 cells, which might be responsible for the down-regulation of melanogenesis. On the other hand, phosphorylation levels of PKA and CREB were decreased by DPHC treatment time-dependently in α-MSH-stimulated B16F10 cells. In addition, we found that DPHC treatment significantly inhibited the phosphorylation of PKA and CREB at 3-12 time points (Figure 4). These results suggest that DPHC can inhibit melanin synthesis by down-regulating PKA and CREB signalling in melanocytes.

3.5 | Effect of DPHC on pigmentation in zebrafish embryos

Zebrafish is a useful and popular vertebrate model system for a variety of depigmentation studies. Zebrafish have melanin pigments on their body surface that allow observation of pigmentation without requiring a complex technology. Therefore, the anti-melanogenic activity of DPHC on zebrafish embryos was investigated. Zebrafish embryos were exposed to either DPHC or arbutin at indicated concentrations and pigmentation on the skin was observed at 72 hpf using a microscope (Olympus, Japan). DPHC significantly decreased the pigment of developing zebrafish embryos dose-dependently (Figure 5A). To quantify

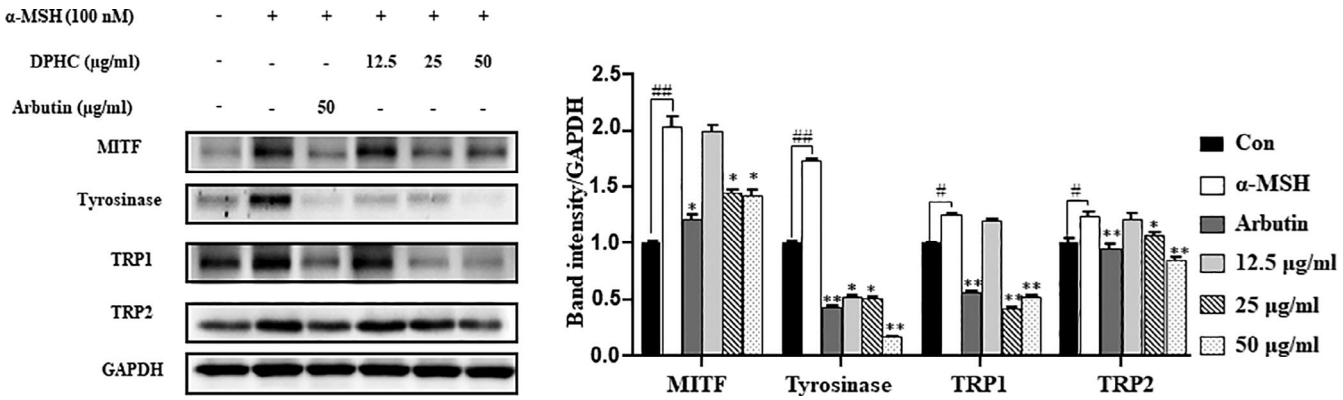

FIGURE 3 Effects of DPHC on expression levels of melanogenesis-related proteins in α-MSH-stimulated murine melanoma cells. Cells were exposed to 100 nM α-MSH in the presence of DPHC at indicated concentrations or 50 μg/mL arbutin. Protein expression levels of MITF, tyrosinase, TRP-1 and TRP-2 were measured by western blot analysis and quantified by Image J. Values are expressed as mean ± SD of triplicate experiments. $^{\#\#}P < .01$, $^{\#}P < .05$ indicate significant differences compared to control group. $^{*}P < .05$ and $^{**}P < .01$ indicate significant differences compared to only α-MSH-treated group

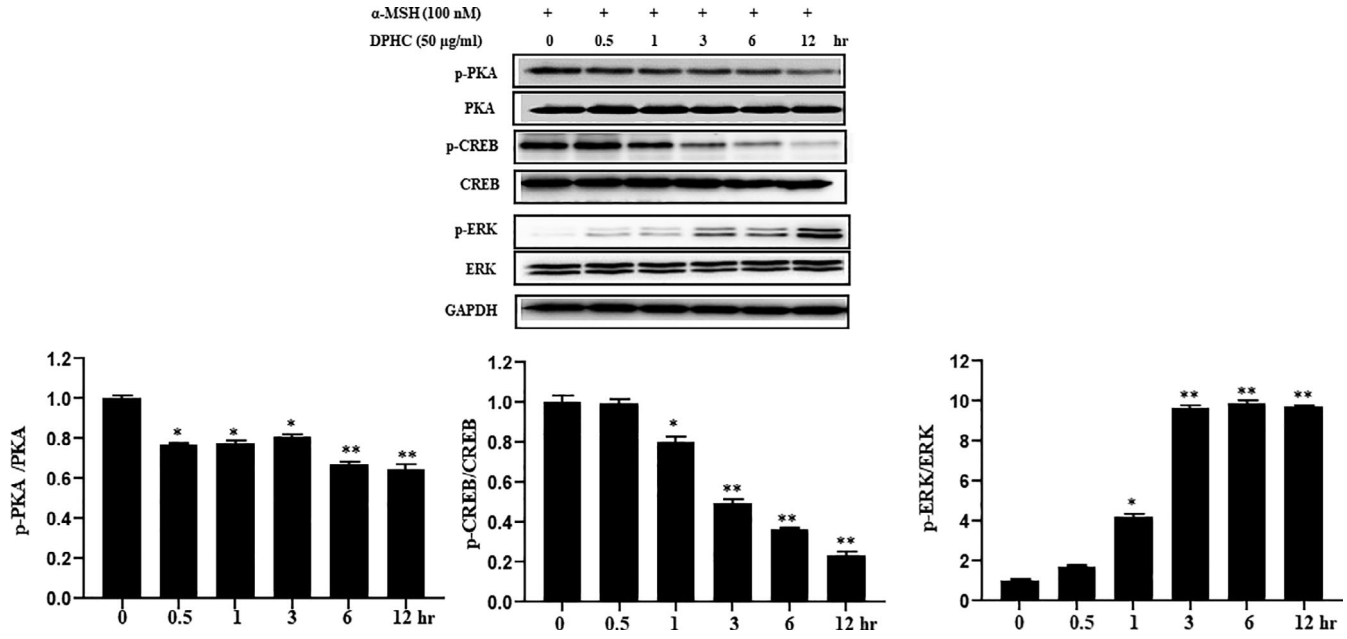

FIGURE 4 Effects of DPHC on PKA/CREB and ERK signalling pathways in α-MSH-stimulated murine melanoma cells. Phosphorylation levels of PKA, CREB and ERK treated with DPHC at indicated concentrations in the presence of 100 nM α-MSH for the indicated time period were determined. Protein expression levels of p-PKA/PKA, p-CREB/CREB and p-ERK/ERK were determined by western blot analysis and quantified by Image J. Equal protein loading was confirmed using a GAPDH antibody. Values are expressed as mean ± SD of triplicate experiments. *P < .05 and **P < .01 indicate significant differences compared to the 0 hour group

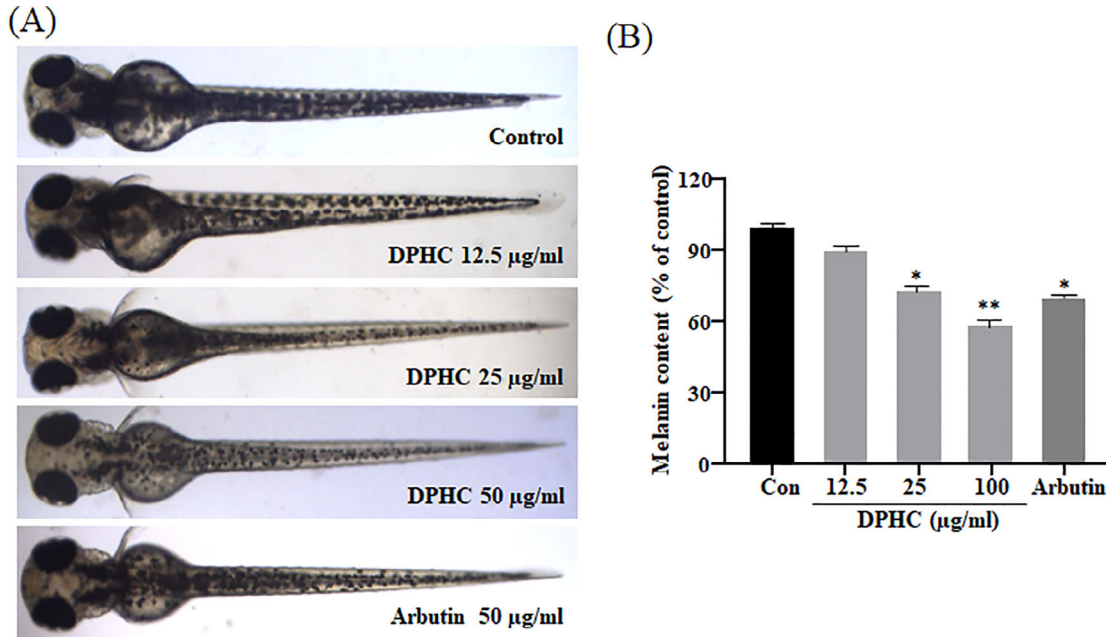

FIGURE 5 Effects of DPHC on pigmentation in zebrafish. Representative photographs of zebrafish A, and relative melanin contents B, are shown. Values are expressed as mean ± SD of triplicate experiments. *P < .05 and **P < .01 indicate significant differences compared to the control group

the pigmentation, melanin contents whole zebrafish embryos extracts were measured. Melanin was distributed the whole body of untreated zebrafish embryo (control). However, DPHC dose-dependently inhibited melanin accumulation compared to the control (Figure 5B). Moreover, at the same concentrations, DPHC was more than arbutin in inhibiting melanin accumulation. As shown in Figure 6, melanin contents in UV-B exposed zebrafish embryos were significantly increased compared to those in non-exposed zebrafish embryos (control).

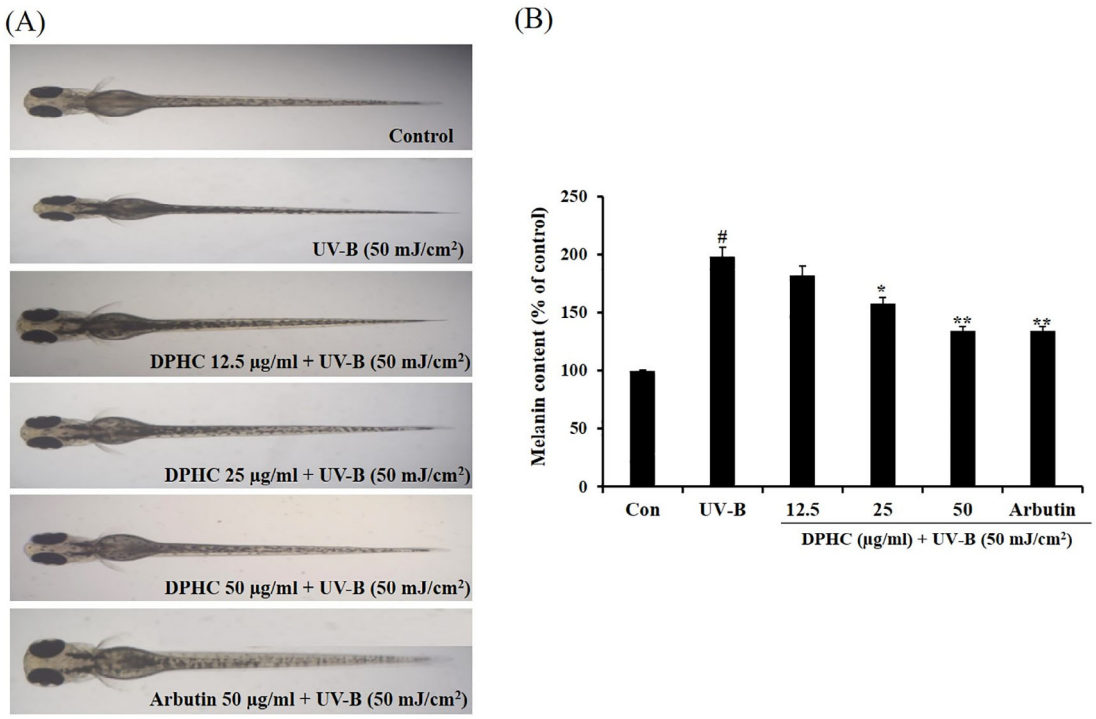

FIGURE 6 Inhibitory effects of DPHC on UV-B-induced hyperpigmentation in zebrafish. Zebrafish embryos were pre-treated with indicated concentrations of DPHC and exposed to UVB (50 mJ/cm^2). Representative photographs of zebrafish A, and relative melanin contents B, are shown. Values are expressed as mean ± SD of triplicate experiments. $^{\#}P < .05$ indicates a significant difference compared to control group. $^{*}P < .05$ and $^{**}P < .01$ indicate significant differences compared to only UV-B-exposed group

However, DPHC decreased melanin contents in UV-B exposed zebrafish embryos in a dose-dependent manner. These results suggest that DPHC can also inhibit UV-B-induced in vivo melanin synthesis. Thus, treatment with DPHC could downregulate melanin formation in zebrafish embryos and UV-B-irradiated zebrafish embryos, leading to decrease of hyperpigmentation.

4 | DISCUSSION

Melanogenesis is a multistage chemical process that leads to the production of skin pigment called melanin, which plays an important role in skin protection from UV exposure.[23] However, immoderate melanin production can lead to darkened skin and hyperpigmentation of skin such as age spots.[24] Several known melanogenesis inhibitors have been used in cosmetic and medical applications to treat skin and pigment abnormalities. However, some of these agents have cytotoxicities or undesirable side effects. For these reasons, a number of studies are currently being conducted to search for naturally derived melanogenesis inhibitors without deleterious side effects. Marine algae as desirability function materials have been widely explored to produce natural bioactive secondary metabolites all over the world. *I. okamurae*, a brown alga, is popular in Korea as a cosmetic ingredient and marine herb due to its wide range of biological activities. Previous studies have proposed that *I. okamurae* can be explored as a potential whitening agent. For example, *I. okamurae* extract has profound inhibitory effects against tyrosinase among 21 species of marine algae.[16] DPHC isolated from *I. okamurae* also has a strong inhibitory effect against mushroom tyrosinase, suggesting the potential of DPHC for suppressing melanogenesis.[16] Based on these results, we wanted to elucidate anti-melanogenesis effects of DPHC at cellular level and in vivo. Therefore, we investigated the effect of DPHC on melanogenesis in α-MSH-stimulated B16F10 melanoma cells and in vivo using a zebrafish model in the present study.

Melanin is produced through a physiological process known as melanogenesis. As a first step to determining the effect of DPHC on α-MSH-stimulated melanogenesis, we investigated whether DPHC could inhibit melanin synthesis in α-MSH-stimulated melanoma cells. We found that DPHC significantly inhibited melanin production without showing cytotoxicity. The decreased extent of melanin content after DPHC treatment at a concentration of 50 μg/mL was more effective that treatment with arbutin (50 μg/mL), which was used as a positive control due to its significant anti-melanogenic effect. Melanin production directly correlates with the activity of tyrosinase and the protein levels of tyrosinase.[25] Therefore, we next evaluated whether DPHC could inhibit intracellular tyrosinase activity in α-MSH-stimulated melanoma cells. DPHC significantly inhibited intracellular tyrosinase activity. Present results showed that DPHC had a strong inhibitory effect against mushroom tyrosinase and decreased the activity of intracellular tyrosinase. It has been reported that the presence of a hydroxyl group and of an electron donator group in the phenol ring is a primary requirement to effectively serve as a tyrosinase substrate.[26]

Many studies have shown that polyphenolic compounds from natural products possess strong tyrosinase-inhibiting activities.[17,18,27,28] Considering these results, the potency as an electron donor and the number of hydroxyl groups as characteristics of the DPHC structure would be key determinants of its inhibitory effect against tyrosinase.

MITF is a major transcription factor of melanogenesis.[29] In general, α-MSH potently induces MITF expression, which can transcriptionally stimulate tyrosinase, TRP-1 and TRP-2 expression, thus increasing melanin production.[30] Therefore, it is important to verify the inhibitory effect of DPHC on melanogenesis by evaluating the down-regulation of the expression of MITF and TRPs. We confirmed that DPHC significantly suppressed the expression of MITF, tyrosinase, TRP1 and TRP2 in α-MSH-stimulated melanoma cells. Results of preceding studies and the present study demonstrated that DPHC could directly inhibit tyrosinase activity and down-regulate the expression of MITF, tyrosinase and TRPs, ultimately leading to decreased melanin production.

In the process of melanogenesis in mammals, UV exposure can stimulate the secretion of α-MSH, which increase the activity of PKA.[28] It has been reported that PKA subsequently upregulates the phosphorylation of CREB that positive regulates the expression of MITF.[31] In the present study, DPHC significantly suppressed the expression of MITF. To illuminate possible mechanisms involved in the reduction of MITF expression induced by DPHC, we evaluated protein levels of PKA and CREB, a key transcription factor involved in the MITF expression. Results of the present study showed that DPHC inhibited PKA and CREB activation which positive regulated MITF expression. These results suggest that DPHC can inhibit melanin synthesis through PKA and CREB signalling pathways by mediation the inhibition of MITF, subsequently downregulating expression levels of TYR, TRP-1 and TRP-2 in α-MSH-stimulated B16F10 melanoma cells. ERK activation can induce the degradation of MITF, which subsequently decreases the expression of tyrosinase.[32] In addition, numerous natural compounds can inhibit melanogenesis through the activation of the ERK pathway.[18,28,33] Therefore, we further examined the influence of DPHC treatment on the activation of ERK in α-MSH-stimulated B16F10 melanoma cells to understand other molecular mechanisms involved in melanogenesis inhibition by DPHC. Data showed that the activation of ERK was promoted after DPHC treatment. This finding suggests that DPHC may inhibit melanin synthesis through degradation of MITF from acceleration of ERK.

Furthermore, we investigated effects of DPHC on pigmentation in vivo using a zebrafish model. Zebrafish (*Danio rerio*) is a small tropical freshwater fish. It has emerged as a useful vertebrate model organism because of its small size, large clutches, transparency, low cost maintenance and morphological and physiological similarities to mammals.[34] Recently, the value of zebrafish as a model organism and a highly useful tool in the discovery of novel whitening agents studies has been recognized.[21,33,35,36] Therefore, zebrafish is a suitable in vivo model for phenotype-based screening and evaluation of melanogenic regulatory compounds. In the present study, DPHC exhibited potent inhibitory effect on pigmentation of zebrafish in vivo, which was comparable to arbutin. We also found that DPHC inhibited melanin synthesis in UV-B-induced zebrafish embryos. These results confirmed that DPHC also could effectively suppress the production of UV-B-induced melanin in vivo and inhibit pigmentation of zebrafish in vivo. Therefore, one interesting finding of our study is the consensus between results obtained from cellular and in vivo experiments.

In conclusion, the present study showed for the first time that DPHC could inhibit a-MSH-stimulated melanogenesis via PKA/CREB and ERK pathway in melanoma cells. It also could inhibit pigmentation in vivo in a zebrafish model. This evidence suggests that DPHC has potential as a skin-whitening agent. Taken together, DPHC could be considered as a novel anti-melanogenic agent to be applied in cosmetic, food and medical industry.

ACKNOWLEDGEMENTS

This research was supported by a grant (NRF-2018R1D1A1B07046262) of the Basic Science Research Program through the National Research Foundation (NRF) funded by the Ministry of Education, Republic of Korea. It was also supported by Soonchunhyang University Research Fund.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

There is no other data available for this article. All the data have been used in the figures.

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How to cite this article: Ding Y, Jiang Y, Im ST, Myung S, Kim H-S, Lee S-H. Diphlorethohydroxycarmalol inhibits melanogenesis via protein kinase A/cAMP response element-binding protein and extracellular signal-regulated kinase-mediated microphthalmia-associated transcription factor downregulation in α-melanocyte stimulating hormone-stimulated B16F10 melanoma cells and zebrafish. *Cell Biochem Funct*. 2021;1–9. https://doi.org/10.1002/cbf.3620