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A dual mechanism of 4-hydroxy-5-methyl-3[2H]-furanone inhibiting cellular melanogenesis

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Synopsis

In previous studies, 4-hydroxy-5-methyl-3[2H]-furanone (HMF) was shown to have potent antioxidative and antimelanogenic effects, suggesting its potential use as a depigmenting agent. The present study investigated its mechanism of action on murine melanoma B16F10 cells stimulated by theophylline, an activator of the cyclic AMP/protein kinase A signaling leading to tyrosinase gene expression. When the cells were stimulated with theophylline, there were dose-dependent increases in cellular tyrosinase protein content and melanin formation, as expected. HMF inhibited the theophylline-stimulated melanin formation as effectively as arbutin, one of the most widely used depigmenting agents in cosmetics. HMF appeared to reduce tyrosinase mRNA and protein content in the cells stimulated by theophylline, indicating it inhibited tyrosinase gene expression. HMF also effectively inhibited tyrosinase-catalyzed melanin formation from dihydroxyphenylalanine in the cells as well as *in vitro.* Therefore, the antimelanogenic effects of HMF were best explained by a dual mechanism inhibiting tyrosinase gene expression and the enzyme activity of pre-existing tyrosinase.

INTRODUCTION

In a classical model of melanogenesis, α-melanocyte-stimulating hormone (α-MSH) stimulates adenylate cyclase, producing cyclic AMP through the G protein-coupled melanocortin 1 receptor (1). Cyclic AMP activates protein kinase A and CREB (cAMP responsive element binding protein) transcription factor, leading to an increase of MITF (microphthalmia associated transcription factor) (2). Binding of MITF to the gene promoters of melanogenic enzymes induces their gene expression (3). Newly synthesized tyrosinase protein undergoes maturation and activation by multiple mechanisms including copper binding, glycosylation, and phosphorylation (4–6). The active tyrosinase then

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catalyzes the oxidation of tyrosine and dihydroxyphenylalanine (DOPA), subsequently leading to the formation of melanin (7).

Skin pigmentation triggered by pathophysiological or environmental factors has a great cosmetic relevance and has prompted the screening of effective natural or chemical agents for decreasing melanogenesis (8). The potential depigmenting agents so far identified include inhibitors of tyrosinase gene expression (9,10) and inhibitors of tyrosinase enzyme activity (11–13). In our previous studies, 4-hydroxy-5-methyl-3[2H]-furanone (HMF) has been isolated from pine needles as an antioxidant component and has been shown to inhibit tyrosinase activity, suggesting its potential use as a depigmenting agent (14,15).

In an attempt to clarify the action mechanism of HMF, the present study investigates its effects on melanin formation and tyrosinase expression in the murine melanoma B16F10 cells stimulated with theophylline, an activator of cyclic AMP/protein kinase A signaling, leading to tyrosinase gene expression. It also examines if HMF affects tyrosinase-catalyzed DOPA oxidation *in vitro* and *in situ* in the cells. The results suggest that HMF may inhibit cellular melanogenesis through a dual mechanism inhibiting tyrosinase gene expression and enzyme activity of the pre-existing tyrosinase.

MATERIALS AND METHODS

REAGENTS

HMF, arbutin, theophylline, and DOPA were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for tyrosinase and β-actin were purchased from Santa Cruz Biotech (Santa Cruz, CA) and Sigma-Aldrich, respectively.

CELL CULTURE AND MELANIN MEASUREMENT

Murine melanoma B16F10 cells (CRL-6475) were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Typically, cells cultured in 24-well culture plates were treated with HMF or arbutin at 50 ~ 400 μM for 60 minutes prior to stimulation with 1.0 mM theophylline for 72 hours. After incubation, the extracellular melanin content in the cell culture media was observed at 490 nm using a multi-well scanning spectrophotometer. Cell morphology and pigmentation were examined under an inverted-phase microscope (Eclipse TS100) from Nikon (Melville, NY). Cell viability was assayed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

PREPARATION OF CELL LYSATES AND WESTERN BLOTTING

After treatment, cells were washed twice with ice-cold PBS and yielded into a lysis buffer (10 mM Tris-Cl, pH 7.4, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 1 mM EDTA, and 0.5% Triton X-100 and protease inhibitor cocktail) in a pre-cooled tube. After 45 minutes of lysis on ice, the homogenate was centrifuged at 14,000 × g for 15 minutes

at 4°C to obtain clear cell lysates. Western blotting was performed as previously described (16). Aliquots of the cell lysates (20 μg protein each), diluted in a Laemmli buffer containing 2% SDS and 1.2% dithiothreitol, were heated at 95°C for five minutes to denature the proteins. The proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel at 200 V for 1.5 hours and transferred to a nitrocellulose membrane. The membrane was incubated with a primary antibody overnight at 4°C, and then with a secondary antibody conjugated with alkaline phosphatase for one hour at room temperature. The bands were detected using a chemiluminescence kit (Pierce, Cheshire, UK) according to the manufacturer's instruction, and were subjected to densitometry analysis.

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

PCR primer sets specific for mouse tyrosinase and D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer3 software (Whitehead Institute/MT Center for Genome Research) on the basis of sequences deposited in the NCBI GenBank database. The sequences of the primers used in this study were: tyrosinase (GeneBank accession number, NM011661) 5′-ATG GGT CAA CAC CCA TGT TT-3′ (sense) and 5′-GGC AAA TCC TTC CAG TGT GT-3′ (antisense); and GAPDH (GeneBank accession number, NM008084), 5′-TGT TCC TAC CCC CAA TGT GT-3′ (sense) and 5′-TGT GAG GGA GAT GCT CAG TG-3′ (antisense). Total cellular RNA was isolated using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. RT-PCR was performed using GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA) in a reaction mixture (20 μl) containing *Maxime* RT-PCR PreMix (iNtRON Biotechnology, Seongnam, Korea), 500 ng RNA, and 20 pmole of gene-specific primer sets. A reverse transcription reaction for the first strand cDNA synthesis was performed at 45°C for 30 minutes, followed by heat inactivation of reverse transcriptase at 95°C for five minutes. A PCR reaction was carried out for 32 cycles of 45 seconds at 95°C, 45 seconds at 55°C, and 60 seconds at 72°C, with a final extension step of five minutes at 72°C. Preliminary PCR runs ascertained that the reactions were in the exponential phase suitable for the quantitative analysis of tyrosinase and GAPDH mRNA. Amplification products were electrophoresed in a 1.2% agarose gel with a 100 bp DNA ladder as a size marker. The gel was ethidium bromide-stained and the band intensities were quantified using a Gel Doc system (BioRad, Hercules, CA).

ASSAY OF TYROSINASE ACTIVITY *IN VITRO* AND IN THE CELLS

The *in vitro* tyrosinase activity was determined by measuring the oxidation of DOPA to DOPA chrome (17). Briefly, the reaction mixture (200 μl) containing cell lysates as a tyrosinase enzyme source (20 μg protein), test materials at different concentrations (0 ~ 1600 μM), and 1.0 mM DOPA as a tyrosinase substrate in 0.1 M sodium phosphate buffer (pH 6.8) was incubated at 37°C for 60 minutes. The DOPA chrome formation was determined at 490 nm. To assess the *in situ* tyrosinase activity, the cells were supplied with 1.0 mM DOPA and incubated for 48 hours. The cell pigmentation was monitored under a microscope.

STATISTICAL ANALYSIS

Statistical analysis was performed by *t*-test. The $p < 0.05$, based on at least three or more independent experiments, was considered to be statistically significant.

RESULTS AND DISCUSSION

To stimulate cyclic AMP/protein kinase A signaling leading to tyrosinase gene expression, the present study used theophylline, a well known inhibitor of cyclic AMP phosphodiesterase that catalyzes cyclic AMP breakdown. As shown in Figure 1A, theophylline treatment of the murine melanoma B16F10 cells stimulated melanin synthesis in a dose-dependent manner. When the lysates were analyzed by Western blotting, there was a theophylline dose-dependent increase of tyrosinase protein content, while the content of β-actin, which was used as a control, was not altered significantly (Figure 1B). The results conform to the notion that cyclic AMP/protein kinase A signaling plays a role in the regulation of melanogenesis by inducing tyrosinase gene expression.

To examine the antimelanogenic effects of HMF, the melanoma cells were pretreated with HMF before the theophylline stimulation. As a positive control, the study used arbutin, a hydroquinone derivative that is widely used in cosmetics as a depigmenting agent (12,18). As shown in Figure 2A, theophylline treatment induced a significant accumulation of dark pigments inside the cells, but this change was prevented by pretreatment with 200 μM of HMF or arbutin. The inhibitory effects of HMF on the cellular melanogenesis appeared to be as strong as those of arbutin (Figure 2B,C). HMF and arbutin had no significant effects on cell viability at the concentrations used in this study (data not shown).

Next, the potential effects of HMF on tyrosinase gene expression were examined. First, the tyrosinase protein content was determined by Western blotting. As shown in Figure

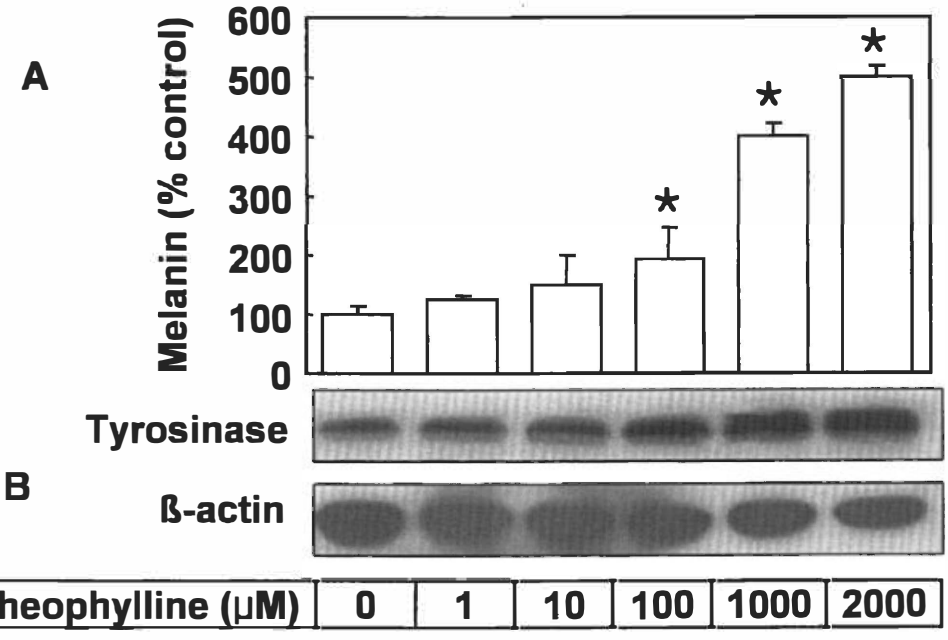

Figure 1. Theophylline stimulates cellular melanogenesis. The cultured murine melanoma B16F10 cells were treated with vehicle or various concentrations of theophylline for 72 hours. The melanin content in the cell-free culture media is expressed as % of vehicle control (A). Data represent mean ± SEM (n = 3). *$p < 0.05$ vs vehicle control. Cell lysates were analyzed for tyrosinase and β-actin by Western blotting (B). Blots shown are representative of at least three independent studies.

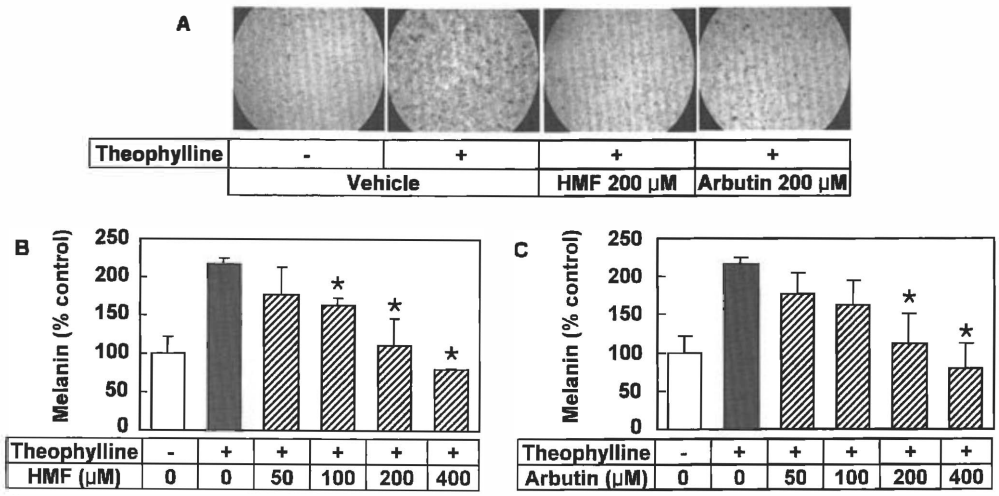

Figure 2. Inhibitory effects of HMF on the cellular melanogenesis stimulated by theophylline. B16F10 cells were pretreated with HMF or arbutin for 60 minutes and then stimulated with 1.0 mM theophylline for 72 hours. Cell pigmentation was observed under microscopy (A). Cell images shown are representative of at least three independent studies. The melanin content in the cell-free culture media is expressed as % of vehicle control (B and C). Data represent mean ± SEM (n = 3). *$p < 0.05$ vs theophylline only.

3A, HMF attenuated the theophylline-dependent increase of tyrosine protein, suggesting that HMF might inhibit melanogenesis by involving down-regulation of the tyrosinase protein content. Arbutin had no inhibitory effect on the tyrosinase protein level (Figure 3B), as has been previously reported (18).

HMF could have decreased the tyrosinase protein level either by inhibiting gene expression or by increasing protein degradation. To address this issue, the next experiment examined whether HMF inhibited tyrosinase mRNA expression stimulated by theophylline. Total cellular RNA was extracted from the treated cells and subjected to RT-PCR analysis for tyrosinase and housekeeping GAPDH mRNAs. The tyrosinase and GAPDH gene-specific primers designed in the present study successfully amplified the predicted PCR products of 564 bp and 396 bp, respectively, from the total cellular RNA, as shown in Figure 3C. HMF appeared to lower the tyrosinase mRNA level in a dose-dependent manner (Figure 3C) in agreement with its effects on the tyrosinase protein content (Figure 3A). The GAPDH mRNA level remained virtually constant (Figure 3C). Therefore the antimelanogenic effects of HMF could be attributed at least partly to the down-regulation of tyrosinase gene expression.

Previous studies have shown that HMF scavenged 1,1-diphenyl-2-picrylhydrazyl free radicals (SC_{50} = 3.0 μg/ml) and inhibited the autoxidation of linolenic acid (IC_{90} = 32 μg/ml) more effectively than ascorbic acid (SC_{50} = 3.3 μg/ml; IC_{90} = 124 μg/ml) (14). HMF also inhibited DOPA autoxidation (IC_{50} = 60 μg/ml) and the catalytic activity of tyrosinase of mushroom origin (IC_{50} = 100 μg/ml) more effectively than arbutin (IC_{50} > 300 μg/ml in both cases) (15). The current study is consistent with these previous findings. As shown in Figure 4A, HMF inhibited the tyrosinase-catalyzed melanin formation from DOPA in cell-free lysates (IC_{50} = 50 μM) much more effectively than arbutin (IC_{50} > 1.6 mM).

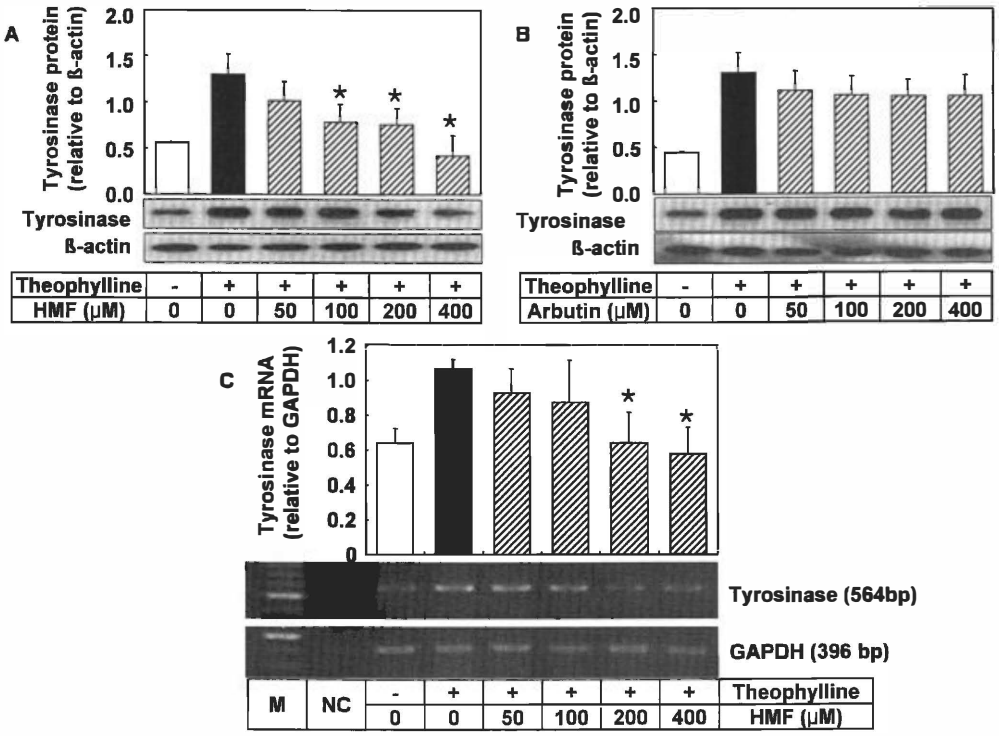

Figure 3. HMF down-regulates tyrosinase gene expression. B16F10 cells were pretreated with HMF or arbutin for 60 minutes and then stimulated with 1.0 mM theophylline for 72 hours. Cell lysates were analyzed for tyrosinase and β-actin proteins by Western blotting (A and B). Blots shown are representative of at least three independent studies. Densitometry was performed to quantify the developed bands, and the graphs show the tyrosinase protein content relative to β-actin. Total cellular RNA was extracted and subjected to RT-PCR analysis of tyrosinase and GAPDH mRNA (C). Stained gels shown are representative of at least three independent studies. M, size marker; NC, negative control without RNA sample. The graphs show the tyrosinase mRNA content relative to GAPDH. Data represent mean ± SEM (n = 3). *$p < 0.05$ vs theophylline only.

The next experiments examined if HMF could inhibit the tyrosinase-catalyzed melanin formation from the DOPA *in situ* in the cells. For this purpose, cells were treated with DOPA after pretreatment with HMF or arbutin, in the absence of theophylline. As shown in Figure 4B, incubation of cells with DOPA alone resulted in the accumulation of a dark pigment inside the cells, providing a chance to determine the effect of test materials on the tyrosinase-catalyzed reactions *in situ* in the cells. As also shown in Figure 4B, the DOPA-dependent cell pigmentation was markedly attenuated by HMF. Arbutin showed a less significant effect, in agreement with the *in vitro* results. Therefore, it is concluded that HMF can interfere with tyrosinase-catalyzed reactions inside the cells.

While genetic background is the most important factor for skin pigmentation (19), other non-genetic factors including hormonal change, chronic inflammation, aging, and ultraviolet light all affect skin pigmentation by stimulating the expression of tyrosinase and other enzymes involved in melanogenesis (2). Tyrosinase inhibitors may be the most non-invasive strategy for the control of skin pigmentation. However, they exhibit less consistent effects *in vivo* (11–13). The inhibitors of tyrosinase gene expression or matu-

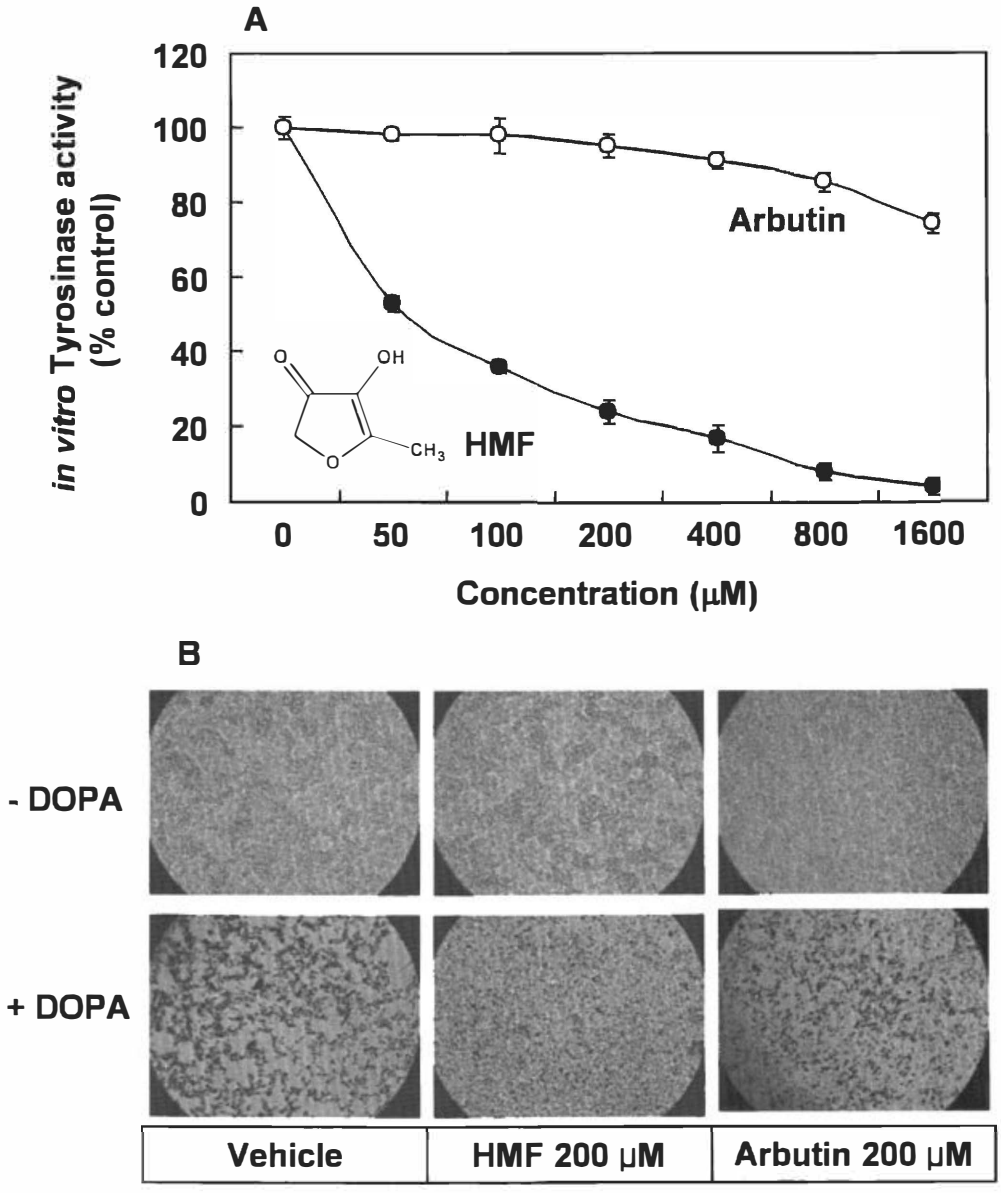

Figure 4. HMF inhibits tyrosinase enzyme activity *in vitro* and *in situ* in the cells. (A), *In vitro* tyrosinase activity was determined using B16 melanoma cell lysates as the enzyme source in the presence of HMF or arbutin at different concentrations. The data are expressed as % of vehicle control and the graph represents mean ± SEM (n = 3). (B) B16F10 cells were pretreated with HMF or arbutin and then supplied with 1.0 mM DOPA or vehicle. Cell images shown are representative of at least three independent studies.

ration thus have potential as alternative strategies for the control of melanogenesis (9,10). HMF is one of the naturally occurring furanones currently used in the food industry as a flavor compound (20,21). The present study demonstrates a novel property of HMF, indicating its interference with multiple steps of cellular melanogenesis and supporting its potential use as a depigmenting agent in cosmetics or medicine.

CONCLUSION

The present study demonstrates that HMF inhibits not only tyrosinase activity (Figure 4) but also its gene expression (Figure 3). This unique dual mechanism of HMF may explain why this compound is so effective in the prevention of cellular melanogenesis (Figure 2). HMF may be useful for the prevention of undesired skin pigmentation.

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