Cyclohexanediol Bis-Ethylhexanoate Inhibits Melanogenesis of Murine B16 Melanoma and UV-Induced Pigmentation in Human Skin

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The role of cyclohexane diester analogues in the formation of melanin has been recently reported. In the present study, we investigated the inhibitory effect of cyclohexanediol bis-ethylhexanoate (CHEH) on melanogenesis in B16 melanoma cells and on UV-B-induced pigmentation in human skin. CHEH significantly reduced the melanin content in a dose-dependent manner, without cytotoxic effects at the effective concentrations. Moreover, CHEH dose-dependently inhibited tyrosinase activity in B16 melanoma cells, as confirmed by Western blot analysis of the tyrosinase protein levels. However, tyrosinase transcript levels remained unchanged under the same experimental conditions. These results indicate that CHEH inhibited melanogenesis in B16 melanoma cells by regulating tyrosinase activity at the post-transcriptional level. On the other hand, in a cell-free system, CHEH did not inhibit tyrosinase activity. This indicated that CHEH suppressed the pigmentation of melanocytes by indirectly regulating tyrosinase activity. Finally, in a clinical trial, a cream containing 1.0% CHEH showed good whitening effect on UV-induced pigmented human skin without adverse effects. In conclusion, we suggest that CHEH may be an effective inhibitor of melanogenesis and useful effects in the treatment of hyperpigmented disorders.

Key words anti-melanogenesis; cyclohexanediol bis-ethylhexanoate; tyrosinase; whitening

Melanogenesis is the process by which melanin—the dark pigment in melanocytes—is synthesized. Melanin determines skin color and protects the skin against UV light. However, abnormal accumulation of melanin results in hyperpigmentary disorders, such as freckles, skin discoloration, and pigmented age spots, which represent serious challenges in dermatology and beauty care. 1,2 Although a number of approaches to control melanin synthesis have been explored, the regulation of melanogenesis is not yet fully understood. 3

Tyrosinase catalyzes the first rate-limiting step in the melanin synthesis pathway. Hence, many natural and synthetic substances have been developed for cosmetic and pharmaceutical purposes as tyrosinase inhibitors. 4 These inhibitors act by regulating tyrosinase catalytic activity, 5,6 mRNA transcription, 7 or maturation. 8 Indeed, unsaturated fatty acids, such as oleic acid, linoleic acid, and α-linoleic acid, have recently been shown to regulate melanogenesis by modifying tyrosinase activity at the post-transcriptional levels. 9

Protein kinase C-β (PKC-β) is also involved in melanogenesis by regulating phosphorylation of the cytoplasmic domain of tyrosinase. 10 It is well known that PKC-β resides in the cytoplasm of melanocytes as an inactive form. Upon activation by diacylglycerol (DAG), activated PKC-β binds the receptor for activated C kinase-I (RACK-I). The activated PKC-β/RACK-I complex translocates to the melanosome, where it phosphorylates tyrosinase. 11 Recently, the effect of cyclohexane diester analogues, which are structurally similar to DAG, on melanogenesis has been reported. 12 One of them, cyclohexanediol bis-ethylhexanoate (CHEH), appears to have a distinguishing inhibitory effect.

In this study, we compared the anti-melanogenic activity of CHEH with that of arbutin and kojic acid, 2 well-known whitening cosmetic agents, and found it to be higher. CHEH-mediated tyrosinase inhibition was examined in vitro. In addition, CHEH toxicity was studied using primary skin irritation tests, and its whitening effects using UV-induced pigmented human skin.

MATERIALS AND METHODS

Synthesis of Cyclohexanediol Bis-Ethylhexanoate trans-1,2-Cyclohexandiol (10 mmol) was dissolved in anhydrous dichloromethane (DCM) (35 mL) and the mixture was cooled at 0 °C. Both 4-dimethylaminopyridine (DMAP) (2 mmol) and trimethylamine (23 mmol) were added as a catalyst and 2-ethyl-hexanoyl chloride (22 mmol) was added slowly. The mixture was reacted for 2 h at room temperature after finishing the addition of the 2-ethyl-hexanoyl chloride. After adding 100 mL of watery hydrochloric acid to reaction mixture, the mixture was extracted with 200 mL of dichloromethane. The dichloromethane layer was dried under reduced pressure and separated by column chromatography on Silica gel with EtOAc–hexanes (1 : 30) solvent mixture as an eluant to give 2.4 g of 2-ethyl-hexanoic acid 2-(2-ethyl-hexanoyloxy)-trans-cyclohexyl ester. The obtained 2-ethyl-hexanoic acid 2-(2-ethyl-hexanoyloxy)-trans-cyclohexyl ester was purified using by fast atom bombardment-mass spectrometry (FAB-MS) and 1H-NMR to obtained 2.4 g purified 2-ethyl-hexanoic acid 2-(2-ethyl-hexanoyloxy)-trans-cyclohexyl ester. (Fig. 1) FAB mass: 369 [M + H]+. 1H-NMR (CDCl3, 300 MHz) δ: 0.88 (t, 6H, J = 6.9 Hz), 0.94 (t, 6H, J = 7.5 Hz), 1.20–1.40 (m, 10H), 1.40–1.70 (m, 8H), 2.05–2.15 (m, 2H), 2.15–2.35 (m, 4H), 2.60–2.70 (m, 4H), 3.00–3.20 (m, 2H), 4.45–4.60 (m, 2H).
(DMSO) was added. The absorbance of the dissolved forma-

The MTT solution was then removed and dimethyl sulfoxide

melanin levels were measured as described previously with

zan crystals was determined at 570 nm by using a microplate

into 96-well plates at 5 × 10^3 cells/well and cultured for 24 h.

were cultured in the presence or absence of different concen-

60°C for 30 min in lysis buffer (10 mM Tris–HCl containing 150 mM NaCl, 2 mM

ethylene glycol bis(2-aminoethylether)-N,N′,N′,N′-tetraacetic acid (EGTA), 2 mM

dithiothreitol, 1 mM sodium orthovanadate, 1 mM

phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). After centrifugation at 12000×g and 4°C

for 10 min, the pellet was used for the melanin content assay. Briefly, the samples were incubated at 80°C for 1 h in 1 mL of

1 M NaOH and then vortexed to solubilize the melanin, after

which the absorbance at 490 nm was measured.

Measurements of Cellular and Cell-Free Tyrosinase Ac-

Activity The cellular tyrosinase activity was assayed by evalu-

ating the oxidation of L-DOPA to dopachrome based on chang-

es in the absorbance at 475 nm.16,17 Briefly, cells were washed

with PBS and then lysed by incubation at 4°C for 30 min in

lysis buffer (10 mM Tris–HCl containing 150 mM NaCl, 2 mM

EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM

phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, and 10 µg/

mL aprotinin). After incubation, the cell lysates were cen-

trifuged at 12000×g for 10 min to obtain the supernatant as the

enzyme source. Next, 100 µg of protein and 2 mM L-DOPA solu-

tion were added to the wells. The plates were then incubated

at 37°C for 30 min, after which the dopachrome formation was

monitored by measuring the absorbance at 475 nm.

Real-Time Reverse Transcription-Polymerase Chain Re-

action (RT-PCR) Total RNA was extracted by using TRIzol ReagentTM (Invitrogen) and resuspended in diethyl pyrocar-

bonate-treated water. The amount and purity of the purified

RNA was spectrophotometrically determined at 260/280 nm.

High quality was confirmed by the presence of intact 18S and

28S detected by means of the Bioanalyzer 2100 (Agilent, CA, U.S.A.). RNA samples were stored at −70°C until use. cDNAs

were synthesized by using 1 µg of total RNA according to the

manufacturer’s protocol (Power cDNA Synthesis Kit; Intron,

Seoul, Korea).

Real-time quantitative PCR was performed by using the ABI Prism 7900 Sequence Detection System (PE Applied Bio-

systems, CA, U.S.A.). Reaction mixtures contained 10 pmol/

µL of each primer and 2× SYBR Green PCR Master Mix (PE Applied Biosystems, CA, U.S.A.), which included the

HotStarTaq DNA Polymerase in an optimized buffer, the
deoxyribonucleotide triphosphate (dNTP) mix (with deoxy-

uridine triphosphates (dUTPs)), the SYBR Green I fluorescent
dye, and the ROX dye (a reference dye). Each of the real-time

quantitative PCR 384-well plates included serial dilutions (1,

1/2, 1/4, 1/8, and 1/16) of cDNA, which were used to generate

standard curves for each of the genes of interest. All primers

were amplified using the same conditions. Thermal cycling

conditions were 50°C for 2 min and 95°C for 10 min followed

by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

In order to exclude the presence of unspecific products, a

curving melt curve analysis was performed routinely after finish-

ing amplification by high-resolution data collection during an

incremental temperature increase from 60°C to 95°C with a

ramp rate of 0.21°C/s. We then converted real-time PCR cycle

numbers to gene amounts (ng) on the basis of the equation.

The real-time PCR analysis was performed on an Applied

Biosystems Prism 7900 Sequence Detection System (PE Applied Bio-

systems, CA, U.S.A.).

The primers for PCR were as follows: tyrosinase, 5′-CTC

TGG GCT TAG CAG TAG GC-3′ (forward) and 5′-GCA AGC

TGT GGT AGT CGT CT-3′ (reverse); tyrosinase-related protein-

1 (TRP-1), 5′-CCC CTA GCA TAT ATC TCC CTT TT-3′

(forward) and 5′-TAC CAT CGT GGG GAT AAT GGC-3′

(reverse); dopachrome tautomerase (DCT), 5′-GTC CTC CAC

TCT TTT ACA GAC G-3′ (forward) and 5′-ATT CGG TTG

Fig. 1. Chemical Structure of Cyclohexanediol Bis-Ethylhexanoate

(CHEH)
after the first application. This assessment took into consideration the elements reported by subjects (subjective and objective signs), as well as those noted by the dermatologist (clinical signs). The frequency, duration, and intensity of signs possibly related to the use of the cream were investigated. Subjective signs included itching, stinging, burning, stiffness, and tightening; objective signs included redness, edema, scale, and papule.

**Western Immunoblotting** Protein levels were determined by western blot analysis following treatment with CHEH for 48 h. Briefly, cultured B16 melanoma cells were washed with PBS, after which they were incubated with lysis buffer (10 mM Tris–HCl containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, and 10 µg/mL aprotinin) on ice for 30 min. After incubation, the cell lysates were centrifuged at 12000×g for 10 min at 4°C. The supernatant was then harvested and used as a protein source for polyacrylamide gel electrophoresis (PAGE). The same amount of protein samples were then denatured and separated by 10% sodium dodecyl sulfate (SDS)-PAGE gels. Next, the proteins were transferred to nitrocellulose membranes (Bio-Rad, Glattburgg, Switzerland) that were subsequently blocked with blocking buffer (5% skim milk in PBS containing 0.1% Tween 20). The proteins were then detected using specific antibodies (dilution 1:250 to 1:1000), after which they were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution). Finally, the bound antibodies were detected using a chemiluminescence horse-radish peroxidase (HRP) substrate (Millipore, MA, U.S.A.) according to the manufacturer’s instructions.

**CHEH-Mediated Whitening of UV-Induced Pigmented Human Skin** To evaluate the usefulness of CHEH in the clinical setting, studies were conducted in agreement with the Declaration of Helsinki and approved by the Ethics Committee of the IEC Korea Skin Research Center (Suwon, Korea), an approved clinical institution. Twenty-two healthy Korean women, aged between 29 and 56 years (mean±S.D., 41.8±4.8 years) were chosen for this study and gave signed, informed consent. On the first day of the study, UV-B radiation emitted from a solar simulator (601 Solarlight, U.S.A.) was applied on the forearm of each of the subjects to determine the minimal erythema dose (M.E.D.). Each subject was then irradiated with UV-B on 2 previously delimited areas (0.8 cm in diameter) on the forearm at an intensity corresponding to 2 M.E.D., as previously determined. Second and third additional UV-B exposures of 0.5–3.0 M.E.D. (depending on each subject’s reaction) were performed on each area after a 2- or 3-d interval. Finally, we obtained a pigmentation equivalent to a decrease in the L value of about 3–4 units. The last exposure was performed about 7 d before the start of repeated applications of the test cream. All subjects applied the cream (with or without 1.0% CHEH) on the tested regions twice a day for 8 weeks as necessary. Assessment of skin color was performed using the CM2600d spectrophotometer (Minolta, Osaka, Japan) before treatment and at 4 and 8 weeks after treatment.

**Safety Evaluation** The safety profile of the applied cream was assessed by a clinical dermatologist at 4 and 8 weeks after the first application. This assessment took into consideration the elements reported by subjects (subjective and objective signs), as well as those noted by the dermatologist (clinical signs). The frequency, duration, and intensity of signs possibly related to the use of the cream were investigated. Subjective signs included itching, stinging, burning, stiffness, and tightening; objective signs included redness, edema,

![Fig. 2. Effect of CHEH on Mouse B16 Melanoma Cell Viability](image)

The cells were seeded in 96-well plates for 24 h and then treated with varying concentrations of CHEH (1.25–20 µg/mL) for 48 h. The cell viability was then determined by using the MTT assay.

**RESULTS AND DISCUSSION**

**CHEH Inhibits Melanogenesis and Tyrosinase Activity in B16 Melanoma Cells** To distinguish the inhibitory effects of CHEH from possible cytotoxic effects on B16 melanoma cells, the concentration range at which CHEH was nontoxic to the cells was determined. Cell viability of CHEH-treated cells was determined by the MTT method. As shown in Fig. 2, CHEH did not exhibit any cytotoxicity on B16 melanoma cells at concentrations up to 20 µg/mL. Therefore, we used CHEH concentrations of up to 20 µg/mL in further experiments.

Melanogenesis inhibition was determined after exposure of B16 melanoma cells to varying concentrations of CHEH. In addition, we determined the extent to which melanogenesis occurred by measuring the intracellular levels of melanin. The results are shown in Table 1. Intracellular melanin was reduced by CHEH in a dose-dependent manner with an IC50 value of 8.9 µg/mL. Surprisingly, the anti-melanogenesis activity of CHEH was higher than that of arbutin (>1000 µg/mL) or kojic acid (449.0 µg/mL), 2 well-known whitening cosmetic agents.

Because tyrosinase plays a leading role in melanogenesis, the effect of CHEH on tyrosinase enzymatic activity was assessed based on DOPA oxidation. B16 cells were cultured in the presence or absence of different concentrations of CHEH for 48 h, after which they were collected and lysed to obtain cellular tyrosinase. Reactions for measuring of enzymatic activity were achieved in PBS (pH 6.8) by using DOPA as an enzyme substrate. With an IC50 value of 13.6 µg/mL, CHEH dose-dependently inhibited cellular tyrosinase activity in B16 melanoma cells (Fig. 3). However, CHEH did not directly inhibit tyrosinase activity in the cell-free tyrosinase activity assay (Fig. 4).

From these results, we confirmed the inhibitory effects of CHEH on melanogenesis. According to Chang,[8] newly developed inhibitors should be compared by using the relative...
inhibitory activity, which is calculated by dividing the IC₅₀ value of the new inhibitor to the IC₅₀ value of kojic acid. Kojic acid, the most intensively studied tyrosinase inhibitor, exhibits a competitive inhibitory effect on monophenolase activity, and a mixed inhibitory effect on the diphenolase activity of tyrosinase. In this study, although CHEH did not inhibit the catalytic activity of tyrosinase, we were able to show that it more potently inhibited (by 500-fold) melanogenesis than kojic acid.

Meanwhile, CHEH had an inhibitory effect on melanin synthesis, but the cellular tyrosiasenase activity was higher at the same concentration. It means that CHEH has another mechanism to reduce melanin contents in addition to inhibition of cellular tyrosinase activity. Therefore, we examined the effects of CHEH on mRNA and protein expression of melanogenic enzymes.

**Effect of CHEH on Tyrosinase, TRP-1, and DCT mRNA and Protein Levels in B16 Melanoma Cells** In order to study the mechanism underlying CHEH-mediated melanogenesis inhibition, we conducted real-time PCR to determine levels of mRNA, and Western blotting to verify the protein levels, in CHEH-treated cells. As shown in Fig. 5, the transcription levels of tyrosinase, TRP-1, and DCT were similar in control and CHEH-treated samples. These results indicate that the inhibitory mechanism of CHEH on melanogenesis is probably not associated with regulation by microphthalmia-associated transcription factor (MITF), a key transcription factor for tyrosinase, TRP-1, and DCT.

However, Western blot results showed that the protein levels of tyrosinase and TRP-1 were significantly reduced in a dose-dependent manner under the same experimental conditions (Fig. 6). Thus, CHEH does not affect tyrosinase mRNA levels dependent manner under the same experimental conditions of tyrosinase and TRP-1 were significantly reduced in a dose-dependent manner under the same experimental conditions of tyrosinase and TRP-1 were significantly reduced in a dose-dependent manner under the same experimental conditions. Also, TRP-1 was known to influence tyrosinase activity by forming a complex and stabilizing tyrosinase in the mouse melanocyte. Therefore, it is likely that the decrease of the TRP-1 by CHEH induces the destabilization and degradation of tyrosinase. Otherwise, CHEH did not affect the protein levels of DCT, which converts Dopachrome to DHICA in the process of melanin synthesis. Although the exact mechanism of CHEH on melanogenesis should be continuously studied, we found CHEH effectively inhibited melanin biosynthesis via the quality control of cellular tyrosinase by reducing the protein expression of TRP-1.

**CHEH-Induced Depigmentation of Hyperpigmented Human Skin** Finally, we investigated the depigmenting effects of CHEH on UV-B-induced hyperpigmentation of human skin. The L value was determined by using the Chromameter CR-400 (Minolta, Japan) at the time of the first visit, and at 28 and 56 d after the start of the treatment. The delta-L values in the test group were significantly improved compared with the control group after 56 d (Fig. 7). This result indicates that the cream containing 1.0% CHEH showed good whitening effect on UV-induced pigmentation of human skin. Moreover, the cream is clinically safe and does not cause any adverse reactions, including erythema, burning, and pruritus, as confirmed by safety testing at days 28 and 56 after topical application.

| Sample | Concentration (µg/mL) | Melanin contents (% control) | IC₅₀ Value (µg/mL) |
|--------|---------------------|-----------------------------|-------------------|
| CHEH   | 2.5                 | 57.0 ± 3.4 **               | 8.9               |
|        | 5.0                 | 54.3 ± 5.4 ****            |                   |
|        | 10                  | 47.6 ± 3.6 **              |                   |
|        | 20                  | 39.1 ± 2.3 ***             |                   |
| Arbutin | 200                | 67.5 ± 3.5 **              |                   |
|        | 1000                | 59.2 ± 4.1 ****            |                   |
| Kojic acid | 40              | 88.3 ± 2.5 *              | 449.0             |
|        | 200                 | 57.1 ± 2.4 **              |                   |
|        | 1000                | 42.6 ± 1.8 **              |                   |

The intracellular melanin was extracted with 1N NaOH containing 10% DMSO from B16 cells and determined by measuring the optical density at 490 nm. Melanin content was calculated from a standard curve of synthetic melanin. The data represent the mean ± S.E. of 3 independent experiments. *p<0.05, **p<0.01, and ***p<0.001 compared to control (Student’s t-test).
application of the cream.

CONCLUSION

Many studies have been carried out to screen and develop tyrosinase inhibitors that act as depigmenting agents. Ando et al. reviewed and classified inhibitors of melanin biosynthesis based on the quality control of tyrosinase, for example, by inhibition of mRNA transcription, the aberration of glycosylation, inhibition of catalytic activity, or acceleration of degradation.25) Here, CHEH, an analogue of cyclohexane diester, reduced the melanin contents and the cellular tyrosinase activity in B16 melanoma cells. Western blotting revealed that CHEH downregulated the levels of tyrosinase protein while real-time PCR analysis showed that CHEH did not downregulate the levels of tyrosinase mRNA in the cells. Therefore, CHEH may inhibit the melanogenesis in B16 cells by regulating cellular tyrosinase activity post-transcriptionally. However, this hypothesis remains to be proved and therefore requires further investigation. In this sense, we are currently studying the effect of CHEH on the glycosylation and degradation of tyrosinase for clarifying its inhibitory mechanism on melanogenesis in detail.

Despite the large number of inhibitors of melanin biosynthesis in vitro, only a few are able to reproduce the skin whitening effects in vivo.26) This gap between in vitro and in vivo studies suggests that a new strategy is needed for discovering new depigmenting agents and for validating their efficacy and safety. In the present study, CHEH melanogenesis inhibition in B16 melanoma cells was 500-fold higher than that of kojic acid. Furthermore, CHEH did not induce any severe adverse reactions in a clinical test. Importantly, it exerts good whitening effect on hyperpigmented human skin. Therefore, we suggest CHEH as a useful and safe additive for hyperpigmented disorders.

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