The Importance of 11 α -OH, 15-oxo, and 16-en Moieties of 11 α -Hydroxy-15-oxo-kaur-16-en-19-oic Acid in Its Inhibitory Activity on Melanogenesis

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Keywords

Melanogenesis · Microphthalmia-associated transcription factor · Tyrosinase · *Pteris dispar* Kunze

Abstract

Cosmetic industries have an interest in exploring and developing materials that have the potential to regulate melanin synthesis in human skin. Although melanin protects the skin from ultraviolet irradiation, excess melanin can be undesirable, particularly on the face where spots or freckles are associated with an appearance of aging. In this study, we found that *ent*-11 α -hydroxy-15-oxo-kaur-16-en-19-oic acid (11 α -OH KA) in *Pteris dispar* Kunze strongly inhibited melanin synthesis by suppressing *tyrosinase* gene expression. The melanogenic transcription factor microphthalmia-associated transcription factor (MITF) is required for this suppression. However, 11 α -OH KA did not modulate the expression level or activity of MITF. Structure-activity relationship analyses suggested that the 11 α -OH, 15-oxo, and 16-en moieties of 11 α -OH KA are essential for the suppression of melanin synthesis. On the other

hand, the 19-COOH moiety is important for preventing cellular toxicity associated with 11 α -OH KA and its related compounds. These results suggest that 11 α -OH KA is an attractive target for potential use in the production of cosmetic items.

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Introduction

Melanin plays a crucial role in protecting the skin from ultraviolet (UV) components of sunlight. In addition, melanin neutralizes free radicals and scavenges toxic drugs and chemicals [1]. However, generation of excess melanin causes visible hyperpigmentation of the epidermis, resulting in freckles, melasma, and age spots [2].

Hyperpigmentation results from an increased number or activity of melanocytes [3]. Melanin synthesis is regulated by tyrosinase (EC 1.14.18.1), which catalyzes hy-

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droxylation of L-tyrosine to 3,4-dihydroxyphenylalanine and its subsequent oxidation to dopaquinone. Dopaquinone is converted to melanin via enzymatic and nonenzymatic reactions [4]. To achieve skin whitening, control of tyrosinase activity and its expression level is important. A number of substances, including linoleic acid [5], hinokitiol [6], kojic acid [7], hydroquinone [8], catechols [9], and salicylate [10], are widely used in cosmetic items where they act as tyrosinase inhibitors.

The regulation of tyrosinase expression levels in melanocytes is a multistep process. First, UV radiation stimulates keratinocytes to synthesize and secrete α -melanocyte-stimulating hormone (α -MSH) [11]. α -MSH then binds to the melanocortin 1 receptor on the melanocyte membrane, which initiates cAMP signaling in the melanocytes. cAMP signaling activates the transcription factor cAMP response element-binding protein (CREB), which induces the melanogenic transcription factor microphthalmia-associated transcription factor (MITF) [12, 13]. In turn, MITF binds to the *tyrosinase* promoter and upregulates the expression of this gene. We have recently identified carnosol and canosic acid from *Callicarpa longissima* as candidates for skin whitening reagents that act by targeting MITF expression [14].

Here, we report that *ent*-11 α -hydroxy-15-oxo-kaur-16-en-19-oic acid (11 α -OH KA), an ingredient in the *Pteris dispar* Kunze leaf extract strongly inhibited melanin synthesis in B16F10 melanoma cells and in a three-dimensional (3D) human skin epidermal model. Although 11 α -OH KA did not alter the expression level or activity of MITF in cAMP-agonist (forskolin, Fsk)-treated cells, it significantly suppressed tyrosinase expression levels. Structure-activity relationship analyses revealed that the 11 α -OH, 15-oxy, and 16-en moieties were essential for melanogenesis inhibitory activity of 11 α -OH KA.

Materials and Methods

Chemicals

Authentic 11 α -OH KA and related kaurens were obtained from BioBioPha (Shanghai, China). Antibodies were obtained as follows: anti-tyrosinase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-MITF (Thermo Fisher Scientific Inc., Waltham, MA, USA), anti-GAPDH (WAKO, Kyoto, Japan), anti-CREB (Genscript, Piscataway, NJ, USA), and p38, pp38, ERK1/2, pERK1/2, and pCREB (Cell Signaling Technology, Danvers, MA, USA). Antigen-antibody interactions were visualized using Chemi Doc XRS (Bio-Rad, Hercules, CA, USA) with the Chemi-Lumi One Super reagent (Nacalai Co. Ltd., Kyoto, Japan) or using the chromogenic reagent immunostain horseradish peroxidase (ATTO Co., Ltd., Tokyo, Japan).

Purification and Analysis of 11 α -OH KA

Fifty grams of dried powder made from the leaves of *P. dispar* Kunze was soaked in 500 mL of methanol, and the resulting extract was passed through 50 g of activated charcoal and was then concentrated to approximately 10 mL (forming viscous precipitates) by evaporation. Viscous precipitates were first washed with 10 mL chloroform and were then re-resolved with 10 mL of methanol to which 500 mL chloroform was added. After removal of unresolved materials by filtration, solvents were re-evaporated. Substances were then concentrated to approximately 20 mL and were applied onto a silica gel column (100 mL; Nacalai, Kyoto, Japan). Melanin-suppressing substances were eluted as 2 peaks when chloroform:methanol (85:15) was used as the eluate. The latter peak fraction was concentrated and applied onto a C₁₈-ODS column (4.6 $\times$ 50 mm, Cosmosil MS-II; Nacalai). The main peak was collected, and substances were recovered in ethyl acetate and re-concentrated by evaporation. The precipitates were then dissolved in 1 mL of ethanol to be hydrolyzed with 9 mL of 1% H₂SO₄-water at 100°C for 30 min. The hydrolyzate was recovered in ethyl acetate, evaporated, suspended in 20% acetonitrile-water, and applied again onto the C₁₈-ODS column. The main peak was collected, and substances were again recovered in ethyl acetate and re-concentrated by evaporation.

Fr. 6 and 6' (hydrolyzed) were purified by recycling HPLC onto an ODS column (15 $\times$ 150 mm i.d., SunFire Prep C18 OBD, Waters; solvent system, 20% acetonitrile/water) to obtain 11 α -OH KA (2.0 mg).

Preparation of 11 α -OH KA Methyl Ester

Synthesis of 11 α -OH KA methyl ester was prepared as described previously [15]. Briefly, 11 α -OH KA (5 mg, 1.6×10^{-5} mol) was reacted with CH₃I (4.1 μ L, 7.5×10^{-5} mol) and K₂CO₃ (10 mg, 6.5×10^{-5} mol) in N, N-dimethylformamide (200 μ L). After being stirred for 14 h at room temperature, 200 μ L dH₂O and 200 μ L ethyl acetate were added, and 11 α -OH KA methyl ester was recovered in the ethyl acetate phase. After removal of ethyl acetate by evaporation, 11 α -OH KA methyl ester was directly confirmed by LC/MS and NMR.

NMR

11 α -OH KA: colorless amorphous powder; ¹H-NMR (CDCl₃ + CD₃OD, 600 MHz) δ : 0.85 (3H, s), 1.14 (3H, s), 2.29 (1H, d, *J* = 12.0 Hz), 2.95 (1H, broad s), 3.93 (1H, d, *J* = 4.6 Hz), 5.16 (1H, s), 5.73 (1H, s); ¹³C-NMR (CDCl₃ + CD₃OD, 600 MHz) δ : 40.5 (C-1), 18.7 (C-2), 37.8 (C-3), 43.4 (C-4), 55.8 (C-5), 19.8 (C-6), 33.7 (C-7), 50.6 (C-8), 62.8 (C-9), 38.8 (C-10), 65.7 (C-11), 65.7 (C-12), 36.8 (C-13), 36.4 (C-14), 210.7 (C-15), 150.3 (C-16), 112.7 (C-17), 28.8 (C-18), 180.4 (C-19), 15.4 (C-20); HRESI-OrbitrapMS *m/z* 333.2066 (calcd for C₂₀H₂₉O₄, 333.2060).

11 α -OH KA methyl ester: colorless amorphous powder; ¹H-NMR (CDCl₃, 600 MHz) δ : 0.76 (3H, s), 1.14 (3H, s), 2.28 (1H, d, *J* = 11.9 Hz), 2.99 (1H, broad s), 3.58 (3H, s, CH₃OCO-), 3.98 (1H, d, *J* = 4.6 Hz), 5.20 (1H, s), 5.81 (1H, s); ¹³C-NMR (CDCl₃, 600 MHz) δ : 41.1 (C-1), 18.8 (C-2), 37.8 (C-3), 43.8 (C-4), 56.0 (C-5), 20.0 (C-6), 33.8 (C-7), 50.6 (C-8), 63.2 (C-9), 38.8 (C-10), 66.3 (C-11), 39.8 (C-12), 36.9 (C-13), 36.5 (C-14), 209.8 (C-15), 150.3 (C-16), 113.0 (C-17), 28.8 (C-18), 177.8 (C-19), 15.4 (C-20), 51.4 (OCH₃); HRESI-OrbitrapMS *m/z* 347.2224 (calcd for C₂₁H₃₁O₄, 347.2217).

Cell Culture

Mouse B16F10 melanoma cells from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, 5.5 mM D-glucose; WAKO), supplemented with 10% fetal bovine serum and antibiotics. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C and were transferred every 2 days via trypsinization to new culture dishes.

Human Epidermal Equivalents (3D Human Skin Model)

Preparation of materials from human source was undertaken through the application and approval of strict ethical principles of the provider Japan Tissue Engineering Co., Ltd., Aichi, Japan (also see http://www.jp-te.co.jp/english/business/ethical_policy.html). The human epidermal equivalents, consisting of multilayered human keratinocytes and melanocytes (LabCyte Melano-Model, Tissue Engineering Co., Ltd., Aichi, Japan), were cultured at 37°C in the manufacturer-supplemented melanogenic medium (with stem cell factor and endothelin 1). Compounds were dissolved in PBS and applied to the surface of the epidermis tissue. The media and compounds were exchanged every 2 days. After 14 days, epidermal equivalents were subjected to melanin measurement and visualization.

Fontana-Masson Staining

Cell layers of 3D human skin models were fixed in 4% paraformaldehyde/PBS for 24 h and embedded in paraffin. Melanin on sections (3 µm thick) was visualized by Fontana-Masson staining.

Assay for Melanin Content

Melanin measurement was performed as described previously [14]. Briefly, the cells were washed twice with phosphate-buffered saline and were recovered in 2-mL collection tubes, followed by centrifugation at 8,000 rpm for 1.5 min. Each cell pellet was suspended in 300 µL of 1 M NaOH and were then lysed by incubation at 45°C for 2 h. Melanin was extracted with a 2:1 chloroform-methanol mixture and was measured with a spectrophotometer (Model 680; Bio-Rad, Hercules, CA, USA) at 405 nm. The protein concentration of the cell pellets was determined using Bradford reagent (Bio-Rad) and was used for normalization of the melanin content.

Quantitative Real-Time PCR

Quantitative real-time PCR analysis, including primer sets, has been described previously [14]. Briefly, total RNA was extracted using an EZ1 RNA Universal Tissue Kit (Qiagen, The Netherlands), and cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). qPCR amplification was performed using EXPRESS SYBR GreenER (Thermo Fisher Scientific). Each of the mRNA expression levels were normalized against the GAPDH mRNA.

Reporter Assay

Procedures of recombinant DNA experiments were approved by the Committee in National Institutes of Biomedical Innovation, Health and Nutrition. The pGL3-mouse *tyrosinase* promoter and mouse *MITF* overexpression vectors have been described previously [14]. To prepare the 3× M-box-luciferase vector, 2 complementary nucleotides (5'-CGC GAA AAG TCA GTC ATG TGC TTT TCA AAA GTC AGT CAT GTG C TT TTC AAA AGT CAG

TCA TGT GCT TTT C and 5'-GAT CGA AAA GCA CAT GAC TGA CTT TTG AAA AGC ACA TGA CTG ACT TTT GAA AAG CAC ATG ACT GAC TTT T) were annealed at room temperature. The DNA fragment was ligated into the *Mlu* I/*Bgl* II site of the pTAL-luciferase vector (Takara, Kyoto, Japan).

To construct the mouse *tyrosinase* promoter with mutation, primers were used in which the M-box region TCA GTC ATG TG was converted to GCG GCC GCG TG using 5' and 3' 20-mer flanking regions. For E-box mutation, the E-box region CAT GTG was converted to GGA TCC. The pGL3-mouse *tyrosinase* promoter vector was amplified using these primers. To construct the double mutant, the M-box mutant vector was amplified using the E-box mutation primers.

Using Lipofectamine 2000 (Thermo Fisher Scientific, Carlsbad, CA, USA), B16F10 melanoma cells plated onto a 24-well plate were cotransfected with the pGL3-mouse *tyrosinase* promoter or pTAL 3× M-box (0.25 µg/well) with the internal reporter pRL-TK (0.05 µg), in the presence or absence of the *Mitf* expression vector (pTarget-mouse *Mitf*, 0.05 µg). After 24 h, the Fsk (20 µM) and 11α-OH KA (1–30 µM) were added, and cells were cultured further for 24 h. Reporter activity was monitored using the Dual Luciferase Reporter Assay Kit (Promega).

Results

Purification of Melanin-Synthesis Inhibitors from *P. dispar*

To identify plant extracts that contain unique melanogenesis suppressors, we collected the leaves of >100 ferns and assessed their ability to inhibit melanin synthesis in Fsk-stimulated B16F10 melanoma cells. Of all plants tested from the fern-library, the extract from *P. dispar* Kunze showed the strongest inhibitory effect on melanin synthesis.

To identify the active inhibiting components of the extract, we first soaked the leaves in methanol and passed the extract through an activated charcoal column. Then, we fractionated the eluent by silica gel chromatography (Fig. 1a) and separated the candidates into 2 fractions (fraction [Fr.] 4 and 6; Fig. 1b). LC-MS analyses suggested that Fr. 6 contained glycosylated compounds composed in Fr. 4. Additionally, the main peak after LC separation (C₁₈-ODS) appears to be composed of at least 2 compounds with a difference of 2 atomic mass units. Because Fr. 4 contained fewer substances than Fr. 6, we tried to separate the main peak in Fr. 6 into the 2 component compounds by recycling column chromatography (Fig. 1c).

After 10 cycles, the 2 compounds were clearly separated, and a thin-layer chromatograph revealed that 1 compound showed strong absorption under UV irradiation (254 nm) (Fr. B) and the other did not (Fr. A) (Fig. 1d).

Fig. 1. Purification of melanin synthesis inhibitors from *P. dispar*. **a** A flow chart of the purification of melanin synthesis inhibitors. **b** Fractions after the silica gel chromatography were evaluated by melanin synthesis inhibition in B16F10 melanoma cells. The cells were incubated with 20 μ M forskolin (Fsk) in the presence of each fraction (1/10,000 dilution) for 48 h and were collected into 1.5-mL tubes. Melanin contents were judged by visual evaluation. Fraction (Fr.) 6 was further separated using HPLC with a C18-ODS column. **c** The main peak in Fr. 6 was collected and applied onto a recycling

HPLC column. After 10 cycles, 2 ingredients were separated as 2 peaks (Fr. A and Fr. B). **d** The ingredients were separated by thin-layer chromatography and visualized by UV (245 nm) (upper image) or by 0.1% sulfuric acid (lower image). **e** Predicted structures of ingredients in Fr. A and Fr. B are indicated. Fr. B shows melanin synthesis-inhibitory activity in B16F10 cells. **f** The main component in Fr. 6 could be hydrolyzed and converted into a hydrophobic product, which was confirmed by the C18-ODS column.

Fig. 2. 11α-OH KA inhibits melanin synthesis in a dose-dependent manner. **a** B16F10 melanoma cells were treated with Fsk (20 μM) for 72 h together with indicated concentrations of 11α-OH KA. **b** The melanin content was measured after normalization by protein concentration ($n = 3$, means and SD are indicated) * $p < 0.05$ compared with Fsk only. **c** 11α-OH KA or kojic acid dissolved in PBS was added onto the surface of a 3D human skin model com-

posed of human primary melanocytes and multilayer keratinocytes. After 14 days (with medium and test compounds changed every 2 days), incubation of the melanin deposition in the 3D human skin model was evaluated under a microscope with low (upper image) and high (lower image) magnification. **d** Melanin content in the 3D human skin model was measured ($n = 3$). * $p < 0.05$ compared with that with PBS only.

MS analyses (Fig. 1e) suggested that these compounds were glycosylated diterpenes. Based on HRESIMS and ^1H -NMR data, which had some impurities, Fr. A was deduced to be *ent*-11α-hydroxy-15-oxokaur-19-oic acid 3-glucoside (11α-OH 16,17-dihydro KA 3-glucoside) [16], whereas Fr. B contained 11α-OH KA 3-glucoside, the enone form of the compound in Fr. A [16]. Only Fr. B was able to inhibit melanogenesis in B16F10 melanoma cells.

Fr. 6 was subjected to hydrolysis to deglycosylate the 11α-OH KA compound. Then, the deglycosylated kaurens were collected by C_{18} -ODS column chromatography (Fr. 6'; Fig. 1f), and 11α-OH KA was separated using recycling column chromatography, and its structure was confirmed to be 11α-OH KA by NMR analyses (see Materials and Methods).

11α-OH KA Inhibits Melanin Synthesis in a Dose-Dependent Manner

To elucidate the mode of action of 11α-OH KA, B16F10 melanoma cells were treated with Fsk for 72 h along with 11α-OH KA (Fig. 2a). Quantification of the melanin content in B16F10 cells showed that 11α-OH KA inhibited melanin synthesis in a dose-dependent manner (Fig. 2b). A 3D human skin model suggested that, despite weak significance, 1 and 3 mM 11α-OH KA more efficiently inhibited melanin synthesis than 1% kojic acid (approximately 70 mM, often included in cosmetic items) (Fig. 2c, d).

11α-OH KA Suppresses Tyrosinase Expression

Since Fsk rapidly induces MITF activity, which then promotes expression of melanogenic enzymes, including

Fig. 3. 11α-OH KA suppresses tyrosinase mRNA and protein levels. **a** Total RNA was extracted from B16F10 melanoma cells that had been treated with Fsk (20 μM for 3 h) together with 11α-OH KA (10 μM) and used as templates for quantitative PCR to measure *Mitf* mRNA expression levels ($n = 2$, means and SD are indicated). **b** Western blot analysis of MITF protein expression in B16F10 melanoma cells treated with Fsk (20 μM) and 11α-OH KA (10 μM)

for 1–8 h. **c** Signal status in Fsk– and 11α-OH KA-treated B16F10 cells was examined by Western blot (~180 min). **d** *Tyrosinase* mRNA levels in B16F10 melanoma cells were measured after treatment for 48 h with Fsk and 11α-OH KA ($n = 2$). * $p < 0.05$, ** $p < 0.01$ compared with that with control (–) (no treatment). **e** Tyrosinase (Tyr) protein levels were evaluated in cells incubated with Fsk for 48 h with indicated concentrations of 11α-OH KA.

tyrosinase, we first examined the effects of 11α-OH KA on MITF expression in B16F10 melanoma cells. Quantitative PCR (Fig. 3a) and Western blot analyses (Fig. 3b) showed that 11α-OH KA had no effect on MITF expression despite unexplainable inhibition of *Mitf* mRNA levels when it is added without Fsk. In addition, activation

(phosphorylation) levels of key molecules, such as CREB, ERK, and p38, at the early phase of the Fsk treatment were not changed by 11α-OH KA (Fig. 3c). On the other hand, both tyrosinase mRNA and protein levels (Fig. 3d, e) were suppressed by 11α-OH KA, possibly independent of the Fsk treatment (mRNA levels in Fig. 3d).

Fig. 4. 11α-OH KA suppresses *tyrosinase* promoter activities. **a** pGL3-mouse *tyrosinase* promoter vector was transformed into B16F10 melanoma cells together with an internal TK reporter. After 24 h, Fsk (20 μM) and indicated concentrations of 11α-OH KA were added to the culture medium, and the cells were incubated further for 24 h ($n = 4$, means and SD are indicated). * $p < 0.05$, ** $p < 0.01$ compared with that with the control (-). **b** The promoter region of the mouse *tyrosinase* gene was mutated ($n = 3$, means and SD are indicated). * $p < 0.05$, ** $p < 0.01$ compared with

that with Fsk(-) or Fsk(+). **c** The expression vector for mouse MITF was cotransformed with the pGL3 mouse *tyrosinase* promoter vector ($n = 3$, means and SD are indicated). * $p < 0.05$ compared with that with Fsk alone. **d** The 3 × M-box-luciferase vector was transformed into B16F10 melanoma cells together with/without the MITF-expression vector. After 24 h, Fsk (20 μM) was added to the culture medium and the cells were incubated further for 24 h ($n = 3$).

11α-OH KA Suppresses Tyrosinase Promoter Activity

To examine whether the reduction of *tyrosinase* mRNA levels was the result of suppression of promoter activities, we performed a reporter assay using the -1.8 kb mouse *tyrosinase* promoter-luciferase vector (Fig. 4a). The *tyrosinase* promoter activity was inhibited by 11α-OH KA in a dose-dependent manner. To rule out the possible contribution of MITF to this suppressed activity, 2 MITF-binding sites (M- and E-box) were disrupted and used in the reporter assay (Fig. 4b). The suppressive effects of 11α-OH KA on the *tyrosinase* promoter were weakened with disruption of the MITF-binding site, particularly with the M-box, and the suppression of 11α-OH KA was completely eliminated with double-site disruption. Given our

observations regarding MITF mRNA and protein levels (Fig. 3a, b), overexpression of MITF created further confusion that the overexpressed MITF prevented 11α-OH KA-induced suppression of *tyrosinase* promoter activity (Fig. 4c).

To verify the role of MITF and the effect of its expression level on 11α-OH KA-mediated suppression of *tyrosinase* promoter activity, we constructed a 3× M-box-luciferase vector (Fig. 4d). Neither the basal (without Fsk) nor the stimulated (by Fsk or MITF overexpression) M-box-derived promoter activities were affected by 11α-OH KA. These results suggest that 11α-OH KA-mediated suppression of *tyrosinase* promoter activity requires MITF. However, 11α-OH KA might not act through

Fig. 5. Structure-activity relationship on melanin-synthesis inhibition. **a** Structures of 11α-OH KA and its related compounds. B16F10 melanoma cells were treated with Fsk (20 μM) for 48 h together with the compounds (10 μM), and cells were collected into 1.5-mL tubes. **b** 11α-OH KA and dihydroxy KA (compound 4) were examined in 3D human skin models (14 days with 1 mM compounds). Photos taken from the surface (upper image: low magnification) and bottom (middle image: high magnification) of the 3D human skin mod-

els are shown. Melanin was visualized by Fontana-Masson staining on vertical sections of the 3D human skin models (lower image). Arrowheads indicate melanin-positive cells. **c** The carboxyl group at the 19 position of 11α-OH KA was converted into methyl ester, and its melanin-synthesis inhibition activity was evaluated in B16F10 melanoma cells (20 μM Fsk for 48 h). **d** The melanin content in the B16F10 melanoma cells was measured (3 μM compounds, 20 μM Fsk; $n = 3$). * $p < 0.05$ compared with that with Fsk only.

modulation of MITF expression levels or posttranslational activation. One possibility is that 11α-OH KA inhibits unknown factors that support MITF recruitment to the *tyrosinase* promoter.

Structure-Activity Relationship of ent-Kaurens on Melanin Synthesis Inhibition

Finally, we examined the structure-activity relationship of 11α-OH KA and its related compounds on the suppressive activities it has: only 11α-OH KA could sup-

Fig. 6. The COOH group at 19 position of 11α-OH KA reduces cell toxicity. **a** B16F10 melanoma cells (1×10^4) were incubated with 11α-OH KA and its related compounds (see Fig. 5a) for 24 h. Cell viability was measured by using cell counting kit 8. Each value at OD450 was normalized by that without compounds (control) ($n = 5$, means and SD are indicated). **b** The same assay was performed with 11α-OH KA, its methyl ester, or rosthornin A (the 19 position is hydroxymethyl) ($n = 5$, means and SD are indicated). * $p < 0.05$, ** $p < 0.01$ compared with that with the control (–).

press melanogenesis (Fig. 1e, Fig. 5a), whereas the other related compounds examined were not, suggesting that the OH group at C-11α, carbonyl group at C-15, and double bond at C-16 are essential for suppression of melanogenic activity. The importance of 11α-OH KA was confirmed in 3D human skin models (Fig. 5b). Both unstained images (surface and bottom) and melanin visualization by Fontana-Masson (vertical section) suggested that 11α-OH KA decreased the number of melanin-containing cells in the 3D human skin models, especially at the basement side.

During purification of 11α-OH KA, we noticed that glycosylation at the C-19 position might not be essential to suppress melanogenesis. To examine the importance of the C-19 position, we modified the COOH group to methyl ester (Fig. 5c). The methylated compound more efficiently suppressed melanogenesis than did 11α-OH KA (Fig. 5d), suggesting that 11α-OH KA methyl ester might be efficiently incorporated into cells, and the COOH group at C-19 was not essential for the melanogenic suppressive activity of 11α-OH KA.

Finally, we reviewed cell toxicity of 11α-OH KA. In B16F10 melanoma cells, 11α-OH KA and its related compounds having the COOH group at their 19 position did not show cell toxic activity up to 30 μM (Fig. 6a). In contrast, 11α-OH KA methyl ester showed cell toxicity at 3 μM. Moreover, rosthornin A that has hydroxymethyl at its 19 position showed high cell toxicity, suggesting that the COOH group at the 19 position reduced cell toxicity of these kaurens.

Discussion

Here, we have shown that 11α-OH KA from *P. dispar* Kunze leaf extract strongly inhibited melanogenesis in mouse B16F10 melanoma cells and 3D human skin model without obvious cellular toxicity.

Originally found in *Pteris semipinnata*, 11α-OH KA was shown to inhibit cell proliferation and induce apoptosis in several cell lines [17, 18]. When human lung cancer A549 cells are treated with 20–80 μg/mL (approximately 60–240 μM) of 11α-OH KA, the induction of the apoptotic factor p21 and activation of caspase-3 are observed [17]. In addition, 11α-OH KA induces mitochondrial mediated apoptosis in human nasopharyngeal carcinoma CNE-2Z cells, which is accompanied by a decrease of Bcl-2 and NF-κB protein levels [18]. However, no other physiologically relevant activity of 11α-OH KA, including suppression of melanogenesis, had been reported prior to the present report, which is the first study on the effect of 11α-OH KA on melanogenesis in mouse and human.

MITF is a key regulator of melanogenic processes and is regulated by a variety of signaling systems at different levels, including gene expression and posttranslational modifications. MITF is phosphorylated by mitogen-activated protein kinase, ribosomal S6 kinase, p38, and GSK3β [19–22]. Phosphorylated MITF can bind to DNA (M-box) more efficiently than its dephosphorylated form. Since 11α-OH KA showed no effect on the MITF expres-

sion level or on its activity on the synthetic M-box-derived reporter, the target of 11 α -OH KA might be effectors of MITF present on the *tyrosinase* promoter. For example, the transcriptional coactivators CBP and p300 are known to interact with the N-terminal transactivation domain of MITF [23, 24] and activate MITF-dependent transcription. Similarly, lymphoid enhancer-binding factor 1 binds to the *tyrosinase* promoter and promotes MITF-dependent *tyrosinase* gene expression [25, 26].

In this study, 11 α -OH KA from *P. dispar* Kunze strongly inhibited melanin production in mouse B16F10 melanoma cells and 3D human skin model. In contrast to the apoptotic activities of 11 α -OH KA and its related kaurens, this melanogenic inhibitory effect highly depended on the specific chemical structure of this compound, suggesting that the melanogenic targets and signaling cascades modulated by 11 α -OH KA might be different from those targeted in its cell death signaling activity. Notably, 11 α -OH, 15-oxy, and 16-en moieties in the *ent*-kauren skeleton are essential for melanogenesis suppression, while the COOH group at the 19 position may contribute to escape from cellular targets that lead to cell death.

In addition to the antimelanogenic activity, we found that 11 α -OH KA had anti-inflammatory activities, such as suppression of cytokine production, in human keratinocytes damaged by UV. To put 11 α -OH KA's beneficial properties into practical use, we first registered "Pteris Dispar Leaf Extract" on the list of International Nomenclature of Cosmetic Ingredients, INCI, and the extract was subjected to human safety studies, including in vitro skin irritation tests using 3D human skin models, genotoxicity tests (Ames tests), in vivo primary skin irritation

test (24-hour closed patch test), cumulative skin irritation and skin sensitization test (Repeated Insult Patch Test, RIPT), and in vitro 3T3 Neutral Red Uptake (NRU) phototoxicity test. Fortunately, the extract, up to 1% (w/w), has been found to have no toxic properties. Coupling with these safety studies, elucidation of the molecular mechanisms by which 11 α -OH KA inhibits melanogenesis is also needed.

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Statement of Ethics

Preparation of materials from human source was undertaken through the application and approval of strict ethical principles of the provider Japan Tissue Engineering Co., Ltd., Aichi, Japan (see http://www.jpte.co.jp/english/business/ethical_policy.html).

Disclosure Statement

A.K. and M.Y. are employees of Momotani Juntenkan Ltd., which paid 6,900,000 JPY, 2015 FY toward the costs of this study. The other authors declare that they have no conflicts of interest.

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