Epidermal hydrating and anti-melanogenic effects of rice-derived glucosylceramides and elasticamide on cell basis evaluation

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Abstract (Maximum of 250 words)

Background: Glucosylceramides (GlcCer) promote skin barrier function and GlcCer mixture exhibit anti-melanogenic effect. Rice GlcCer fraction consists of multiple molecules of GlcCer and Cer including ceramide[AP] (elasticamide). However, no actions of these molecules in skin cells have been reported. Thus, we evaluated the effects of rice-drived GlcCer and Cer on epidermal hydration and melanogenesis.

Methods: GlcCer and Cer were purified from crude rice oil. Hydrating effects on the reconstructed human epidermal keratinocytes (RHEK) were evaluated by measuring transepidermal water loss (TEWL). For active compounds, Cer contents and expressions of moisturizing factors were measured. Anti-melanogenic effect was evaluated in B16 melanoma cells and UV-irradiated human skin. Anti-melanogenic effect and mechanism of active compounds were also evaluated in normal melanocytes.

Results: GlcCer[d18:2(4*E*,8*Z*)] with C18 to C26 fatty acids and elasticamide decreased TEWL. The potential of GlcCer depended on the fatty acid length and the effect of elasticamide was as strong as GlcCer[d18:2(4*E*,8*Z*)/26:0]. Elasticamide increased Cer[NS/NDS] with enhancing GlcCer synthase expression, whereas GlcCer[d18:2(4*E*,8*Z*)/26:0] enhanced filaggrin expression. On melanogenesis,

GlcCer[d18:2(4*E*,8*Z*)] with C18 and C20 fatty acids and elasticamide suppressed melanogenesis. Elasticamide also suppressed melanogenesis and expressions of tyrosinase related protein 1 (TYRP-1) and cellular ATP production in normal melanocytes.

Conclusion: GlcCer exhibited fatty acid length-dependent epidermal hydration with filaggrin expression. Elasticamide showed strong effect with enriching Cer[NS/NDS]. GlcCer[d18:2(4*E*,8*Z*)] with C18 and C20 fatty acids and elasticamide suppressed melanogenesis. Suppression of TYRP-1 and ATP production were involved in the effect of elasticamide. These effects might be involved in the clinical effects of rice GlcCer fraction.

Keywords: glucosylceramide; elasticamide; transepidermal water loss; glucosylceramide synthase; filaggrin; melanin.

Introduction. Skin ceramides (Cer) are sphingolipids mainly existing in stratum corneum (SC) and play pivotal roles on epidermal hydration and barrier function [1] with other lipids and moisturizing proteins such as filaggrin [2]. Especially 12 major Cer exist in intracellular SC lipids [3]. Among Cer, ω-hydroxy Cer which has very long chain fatty acids exhibit stronger barrier function such as Cer[EOS]. [4] In terms of plant-derived major Cer, glucosylceramides (GlcCer) dominantly exist in wide variety of botanical resources [5]. GlcCer are decomposed into glucose and a Cer in intestinal mucosa and then transferred from the intestines through lipid-absorbing pathways and Cer into the veins via the lymph ducts [6]. Cer reach the epidermis and exert moisturizing and barrier functions [7]. Recent studies revealed that GlcCer were directly absorbed from the intestines without glycolytic digestion and reached the lymph nodes [8]. The findings of clinical studies indicate the potential of rice-derived GlcCer to reduce transepidermal water loss (TEWL) [9]. Increases in epidermal ceramide production may be one of the mechanisms by which GlcCer supplementation prevents epidermal dehydration [10].

As other biological effects of GlcCer on skin condition, maize cerebroside mixture has been reported to suppress melanogenesis in B16 melanoma cells [11]. In contrast, intrinsic GlcCer were found to enhance melanogenesis by facilitating the transportation of tyrosinase from the Golgi complex to melanosomes [12]. Therefore, GlcCer are essential for

melanogenesis. In contrast, C2-Cer was also found to suppress melanogenesis by inhibiting DNA synthesis, the Akt/PKB signaling pathway, and tyrosinase activity in melanoma [13].

Rice GlcCer consist of multiple molecules which are composed of different types of sphingoid bases and multiple length of free fatty acids. Beside GlcCer fraction, there are free Cer as minor constituents including elasticamide (ceramide[AP]). However, no study results have been reported regarding epidermal hydrating and anti-melanogenic effects of these single molecules of GlcCer and elasticamide. Therefore, in this study, we have isolated 13 GlcCer and elasticamide (Cer[t:18:0/24:0]) from gummy by-products for rice bran oil and evaluated epidermal moisturizing activity and anti-melanogenic effects.

Materials and Methods.

Preparation of GlcCer and elasticamide

The crude GlcCer and elasticamide fraction manufactured from gummy by-products obtained during the manufacturing process of rice bran oil was used as a starting material. The fraction (10 g) was applied to flash column chromatography equipped with a silica gel column. After washing the column with hexane: ethyl acetate (9:1→0:10) to remove less polar fractions, the mobile phase was changed to chloroform and methanol. The composition of the solvent was gradually changed from chloroform and methanol (10:1→0:10) to obtain a chloroform: methanol (8:2) fraction as the GlcCer fraction. The fraction was separated and repeatedly purified by HPLC equipped with an ODS column. Methanol was used as the solvent. HPLC fractions were evaporated to obtain GlcCer[d18:2(4*E*,8*Z*)/18:0] (**1**, 23.6 mg), GlcCer[t18:1(8*Z*)/20:0] (**2**, 16 mg), GlcCer[d18:2(4*E*,8*Z*)/20:0] (**3**, 117.6 mg), GlcCer[d18:2(4*E*,8*E*)/20:0] (**4**, 56.5 mg), GlcCer[t18:1(8*Z*)/22:0] (**5**, 32.2 mg), GlcCer[d18:1(4*E*)/20:0] (**6**, 6.0 mg), GlcCer[d18:2(4*E*,8*Z*)/22:0] (**7**, 29.1 mg), GlcCer[d18:2(4*E*,8*E*)/22:0] (**8**, 4.5 mg), GlcCer[t18:1(8*Z*)/24:0] (**9**, 5.8 mg), GlcCer[d18:2(4*E*,8*Z*)/24:0] (**10**, 28.7 mg), GlcCer[d18:2(4*E*,8*E*)/24:0] (**11**, 8.9 mg), GlcCer[t18:1(8*Z*)/26:0] (**12**, 8.8 mg), and GlcCer[d18:2(4*E*,8*Z*)/26:0] (**13**, 3.9 mg). The chemical structure of each GlcCer was identified by comparisons of ^{1}H- and ^{13}C- NMR spectra with reference values listed in a previous study [9] (Figure 1).

To obtain elasticamide (**14**), the chloroform and methanol (10:1) fraction was purified by HPLC equipped with an ODS column. A mixture of methanol and tetrahydrofuran (9:1) was

used as the solvent. The chemical structure was identified by ^{1}H- and ^{13}C-NMR spectra with the reference values listed in a previous study [14,15].

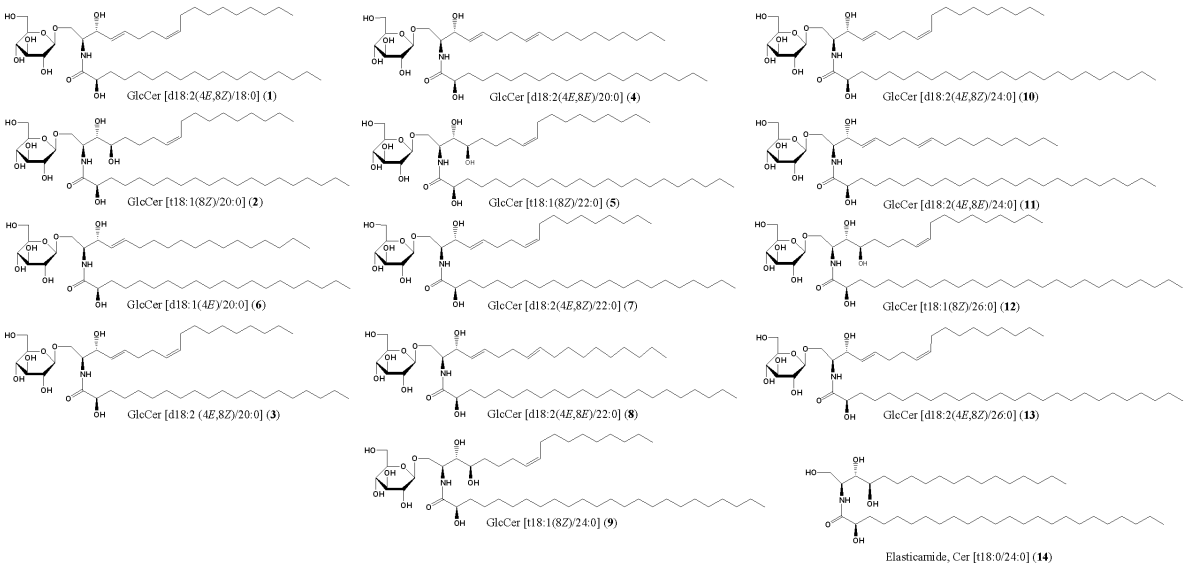

Figure 1. Chemical structures of GlcCer (**1**-**13**) and elasticamide (**14**).

Culture of reconstructed human epidermal keratinocytes (RHEK) and measurement of TEWL

Each cup of the RHEK models were placed onto a 24-well culture plate and assay medium was added under the cup. After incubating the plate for a day, the RHEK models were treated with a solution of GlcCer (10 μM) or elasticamide (1-10 μg/mL). Depending on each experiment, the culture time was selected. Namely, RHEK models were cultured for 7 days for TEWL measurement, 4 days for real-time RT-PCR, and 5 days for ceramide analysis. TEWL was measured by Tewitro TW24 (Courage+Khazaka).

Cer determination and real time RT-PCR in RHEK treated with elasticamide (14)

The whole tissue of the RHEK models were carefully peeled from the membrane. The separated SC samples were washed with PBS and stored at −80 °C until the determination of ceramides. The lipid extraction and thin layer chromatography were performed by previous reported methods [16] as well as quantitative real-time RT-PCR.

Analysis of RHEK treated with GlcCer (13)

In addition to evaluation of TEWL and Cer contents, we conducted electron microscopic analysis on RHEK treated with **13** (10 μM) for 7 days. Protein expression of corneodesmosin and filaggrin were evaluated by immunostaining and western blotting methods.

Evaluation of melanogenesis, cell viability, mRNA expression, cellular ATP production, and tyrosinase inhibitory activity in B16 melanoma cells treated with GlcCer or elasticamide (14)

B16 melanoma cells (5×10^4 cells/mL) were seeded on a 96-well plate (100 μL for the cytotoxicity assay), a 48-well culture plate (200 μL for melanogenesis), and a 24-well culture plate (500 μL for RT-PCR). After an incubation for 24 h, GlcCer or **14** and theophylline (2 mM) were added and incubated for 72 h. After a further incubation, medium was removed from the 48-well plate and distilled water (100 μL/well) was added. Cells were sonicated and 6 N NaOH (20 μL/well) was added. The absorbance of the sonicated solution was measured at 405 nm (reference wavelength: 660 nm). To evaluate cytotoxicity, MTT assay was used. Total RNA was extracted and the mRNA expression of enzymes related to melanogenesis in B16 melanoma cells was assessed by quantitative real-time RT-PCR.

For tyrosinase inhibitory activity, lysate of B16 melanoma cells were used as crude tyrosinase. Crude enzyme solution (21 μg protein/50 μL/well), a 2 mM L-DOPA solution (40 μL/well), and solution of **3** or **14** (10 μL/well) were placed onto a 96-well plate (DMSO final concentration: 1%). The enzyme reaction was performed at 37°C for 90 min. After the enzyme reaction, absorbance was measured at 475 nm (reference wavelength: 660 nm).

Luciferase assay was used for ATP quantification. B16 melanoma cells (1.0×10^5 cells/mL) were seeded on a 96-well plate (100 μL/well) and incubated for 24 h. Medium was then replaced with that containing theophylline (2 mM) and **3** or **14**. After an incubation for 24 h, medium was removed and cells were gently washed with PBS (-). Glo Lysis Buffer (200 μL/well) was added to each well and incubated for 5 min. The bioluminescence of each well was measured using an ATP assay reagent for cells.

Evaluation of cell viability, cellular ATP production, and mRNA expression of factors related to melanogenesis in normal human epidermal melanocytes (NHEMs) treated with GlcCer (3) or elasticamide (14)

NHEMs (2.0×10^5 cells/mL) were seeded on a 24-well plate (500 μL/well) and incubated for a day. Medium was replaced with that containing 100 nM α-MSH and **3** or **14**. After

incubating the plate for 2 days, RNA was extracted from cells using a conventional method and gene expression levels were confirmed by RT-PCR. To evaluate cell viability, NHEMs (5.0×10^4 cells/mL) were seeded on a 96-well plate (100 μL/well). After a pre-incubation for a day, medium was replaced with that containing **3** or **14** and cells were incubated for 2 days. Cell viability was evaluated using the standard method of Cell Counting kit-8.

Evaluation of melanogenesis in 3D-cultured keratinocytes and melanocytes treated with GlcCer (3) or elasticamide (14)

Asian-derived human 3D-cultured melanocytes (MEL-300-A, Kurabo Ind. Ltd.) was used for measurements of melanogenesis. MEL-300-A were pre-incubated for a day. Medium was changed with that containing **3** or **14**, and MEL-300-A were then incubated for 19 days. After the end of the culture period, melanin was extracted and quantified. Briefly, tissues were pooled and homogenized in 1% SDS containing 50 μM EDTA and 10 mM Tris HCl (pH 6.8, 450 μL). The homogenate obtained was supplemented with 5 mg/mL proteinase K (20 μL) overnight (45°C), followed by the addition of 500 mM sodium carbonate (50 μL) and 30% hydrogen peroxide (10 μL) and a further incubation at 80°C for 30 min. The mixture was extracted with 100 μL of a mixture of chloroform and methanol (2:1). After centrifugation of the extract at 10,000×*g* for 10 min, absorbance at 405 nm was measured. Synthetic melanin was treated using the above procedure and a standard curve was constructed to measure the content of melanin. Fontana-Masson staining was used for microscopic observations of melanin synthesis.

Clinical examination of UV-induced pigmentation

This randomized, placebo-controlled, double-blind, parallel-group study (UMIN000042696) was conducted to evaluate the effect of Oryza ceramide® manufactured by Oryza Oil & Fat Chemical Co., Ltd. The total content of GlcCer and the concentration of elasticamide (**14**) were 3.0 and 0.14%, respectively. The compositions of GlcCer and other specifications were previously reported.[9] Active capsules contained 40 mg of Oryza Ceramide® (comprising 1.2 mg of GlcCer and 0.06 mg of elasticamide) and 160 mg of γ-cyclodextrin. Placebo capsules contained 200 mg of γ-cyclodextrin. Inclusion criteria were Japanese male and female adults (20 years or older and 59 years or younger) with a normal skin color or fair skin who expressed concerns about dry skin. First selection criteria were individuals with confirmed minimum erythema dose 1 (MED) among 6 UV irradiated areas. Secondary

selection criteria were individuals with high TEWL on the inside of the forearm. Forty-eight subjects were selected for the present study. Subjects took one appropriate capsule (Oryza Ceramide® or placebo) daily after breakfast for 8 weeks. MED and the minimal tanning dose (MTD) were examined at baseline and after 4 and 8 weeks of the intervention as the primary outcome. UV irradiation was performed on the screening day, baseline day, after 4 weeks, and after 8 weeks. According to references, 6 areas (areas A to F) were irradiated by UV rays from 1074 to 2672.4 mJ/cm^2·s at intervals of 1.2 times.

Results.

Effects of GlcCer (1-5, 7, 9-13) and elasticamide (14) on TEWL in RHEK

As a result of evaluation of TEWL from the surface of RHEK, 10 μM of GlcCer[d18:2(4*E*,8*Z*)] including GlcCer[d18:2(4*E*,8*Z*)/18:0] (**1**), GlcCer[d18:2(4*E*/8*Z*)/20:0] (**3**), GlcCer[d18:2(4*E*/8*Z*)/22:0] (**7**), GlcCer[d18:2(4*E*/8*Z*)/24:0] (**10**), GlcCer[d18:2(4*E*/8*Z*)/26:0] (**13**) and elasticamide (**14**) decreased TEWL by 7-day treatment (Figure 2). Among GlcCer, TEWL was improved depending on the length of fatty acids and **13** exhibited most potent hydrating effect. The hydrating effect of **14** was as strong as **13**.

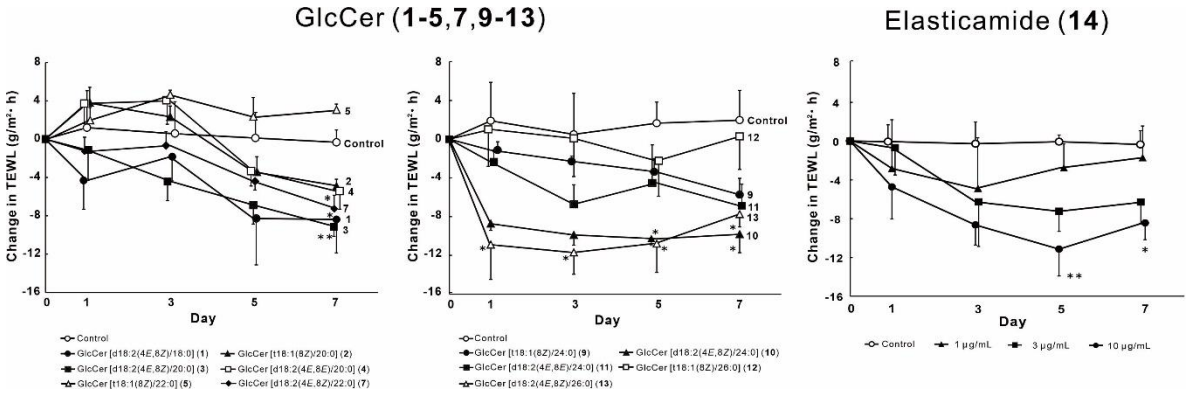

Figure 2. Effect of GlcCer (**1**-**5**, **7**, **9**-**13**) and elasticamide (**14**) on TEWL in RHEK

Each value represents the means ± S.E. (N=3-4). Asterisks denote significant differences from the control group, *p<0.05, ** p<0.01.

Effect of GlcCer (1-5, 7, 9-13) and elasticamide (14) on Cer contents in RHEK

To find the epidermal hydration mechanism, we determined Cer contents in RHEK. However all GlcCer (**1-5**, **7**, **9-13**) had no effect on total Cer contents (Figure 3A). On the other hand, **14** (10 μg/mL, 14.3 μM) significantly increased total Cer and Cer[NS/NDS] (Figure 3B).

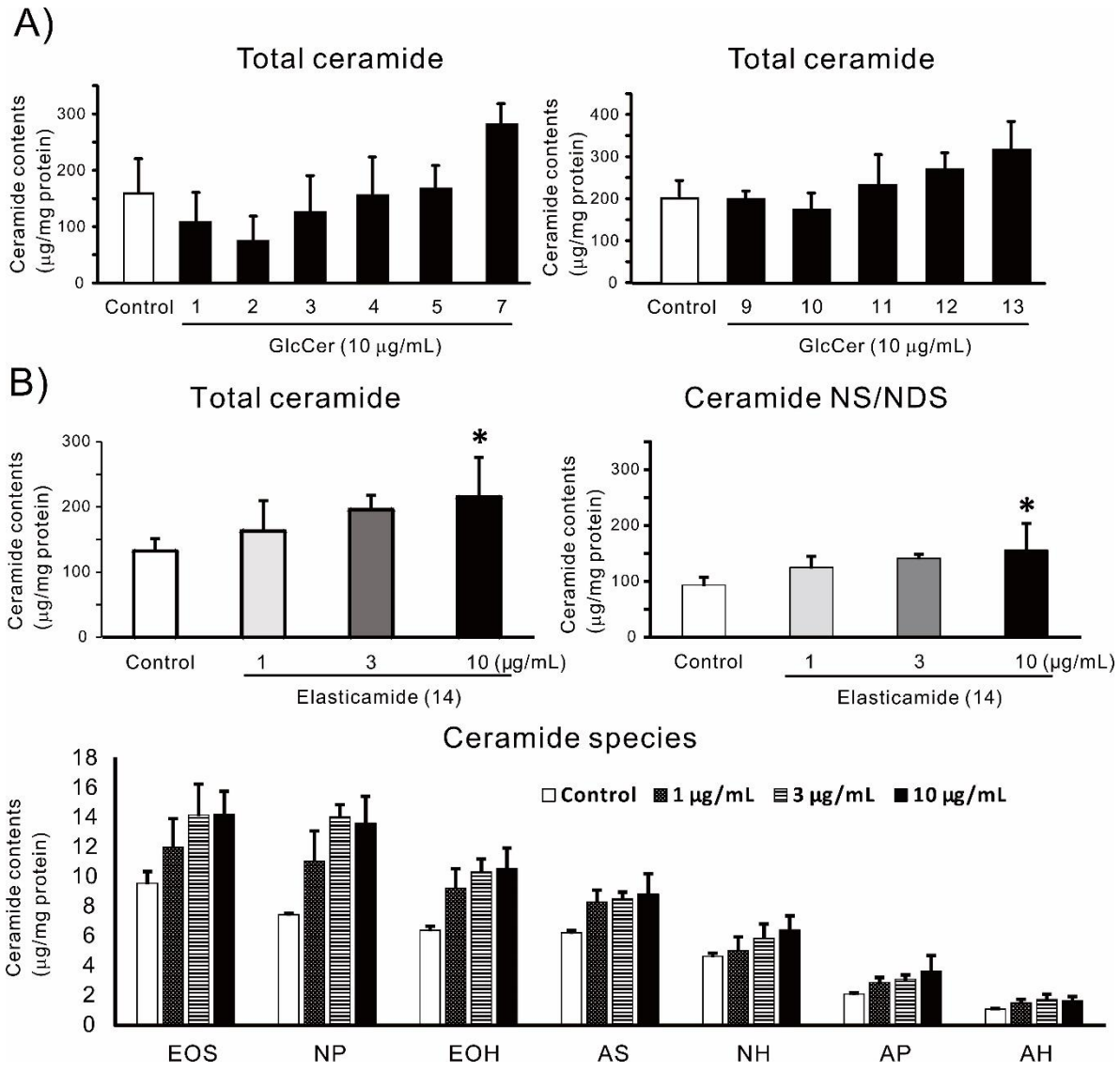

Figure 3. Effect of GlcCer (**1-5**, **7**, **9-13**) and elasticamide (**14**) on Cer contents in RHEK
A) Total Cer in RHEK treated with 10 μg/mL of GlcCer (**1-5**, **7**, **9-13**). B) Changes in Cer species treated with **14**. Each value represents the means ± S.E. (N=4). An asterisk denotes significant difference from the control group, *p<0.05.

Effect of elasticamide (14) on Cer synthesizing enzymes in RHEK

On GlcCer synthase (GCS) expression, **14** significantly enhanced both mRNA and protein expression at 1 μM (Figure 4). mRNA expressions of sphingomyelin synthase (SMS) 2 was also enhanced by **14** (10 μg/mL).

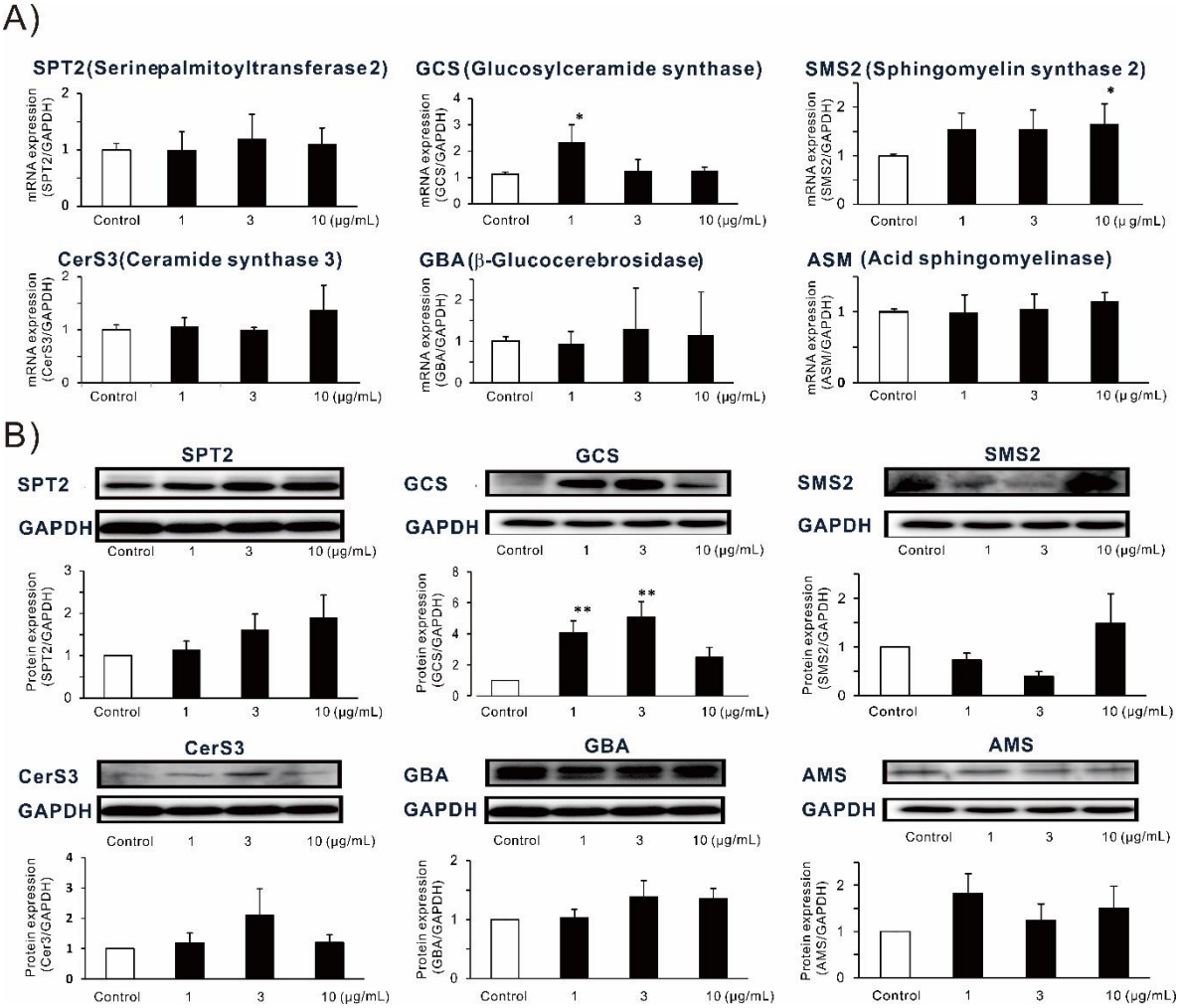

Figure 4. Effect of elasticamide (**14**) on mRNA (A) and protein (B) expression of Cer synthesizing enzymes in RHEK

Each value represents the means ± S.E. (N=4). An asterisk denotes significant difference from the control group, *p<0.05, **p<0.01.

To find the TEWL reduction mechanism of GlcCer[d18:2(4*E*,8*Z*)/26:0] (**13**) which exhibited strongest activity at 10 μg/mL, we performed various evaluation in RHEK. GlcCer

(**13**) dose-dependently decreased TEWL from 1 to 10 μM (Figure 5A). However, **13** had no effect on Cer contents in RHEK (Figure 5B).

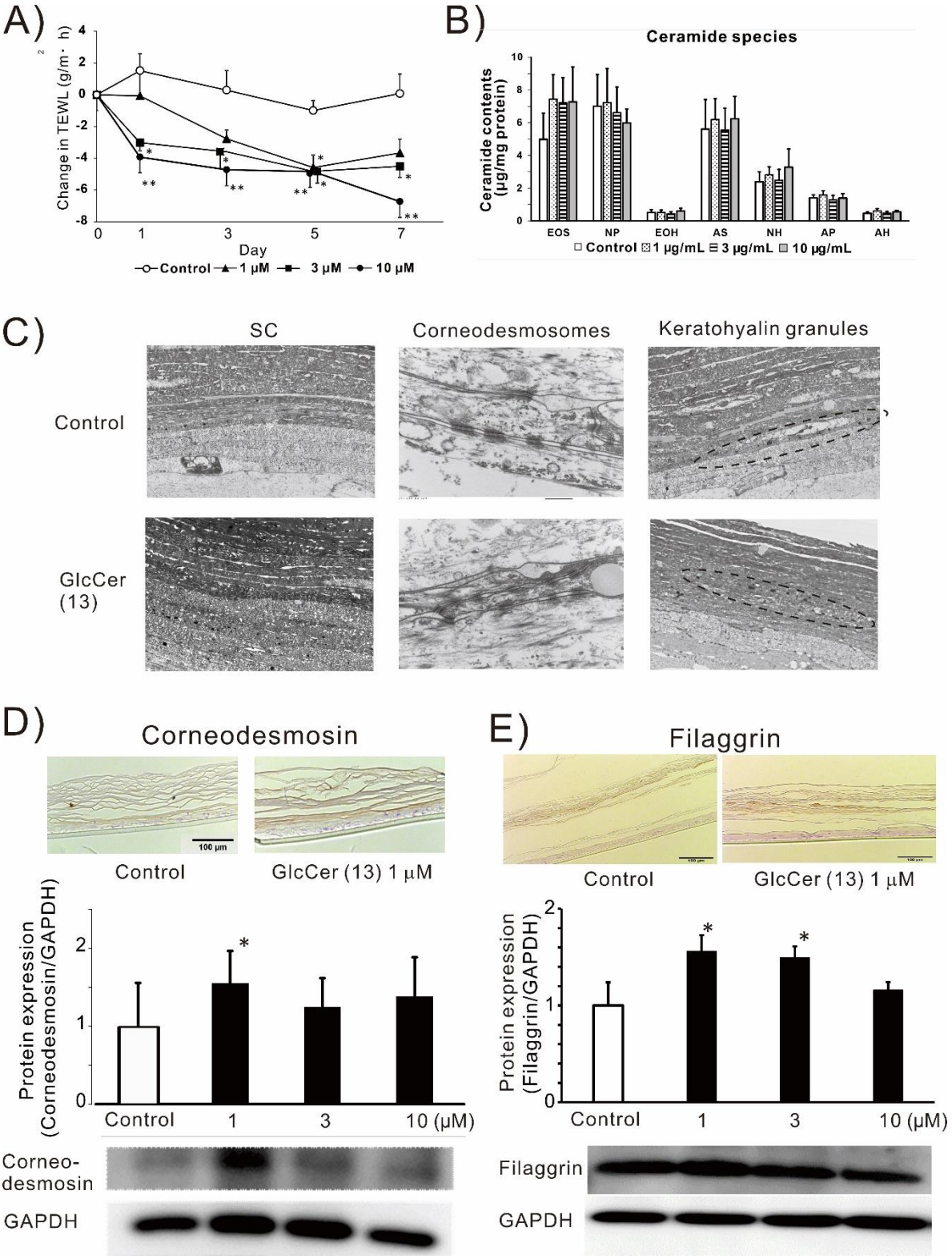

Figure 5. Effect of GlcCer[d18:2(4*E*,8*Z*)/26:0] (**13**) on RHEK

A) Change in TEWL. B) Cer contents after treatment of **13** for 7 days. C) Electron microscopic analysis of cross section images. Expression of corneodesmosomes (D) and filaggrin (E). Each value represents the means ± S.E. (N=4). Asterisks denote significant differences from the control group, *: $p<0.05$, **: $p<0.01$.

On the other hand, electron microscopic observation suggested that **13** changed SC layer denser and increased number and size of keratohyalin granules (Figure 5C). Although **13** did not seem to affect to corneodesmosomes, it enhanced corneodesmosin protein expression (Figure 5D). GlcCer (**13**) also enhanced filaggrin expression at 1 μM (Figure 5E).

Figure 6 shows the possible hydration mechanism of elasticamide (**14**) and GlcCer (**13**). Elasticamide (**14**) increased internal Cer[NS/NDS] synthesize through GCS expression, while GlcCer have no direct effect on skin Cer. However, **13** has changed the SC structure which suggest water retention. Actually, expressions of corneodesmosin and filaggrin were enhanced by **13**.

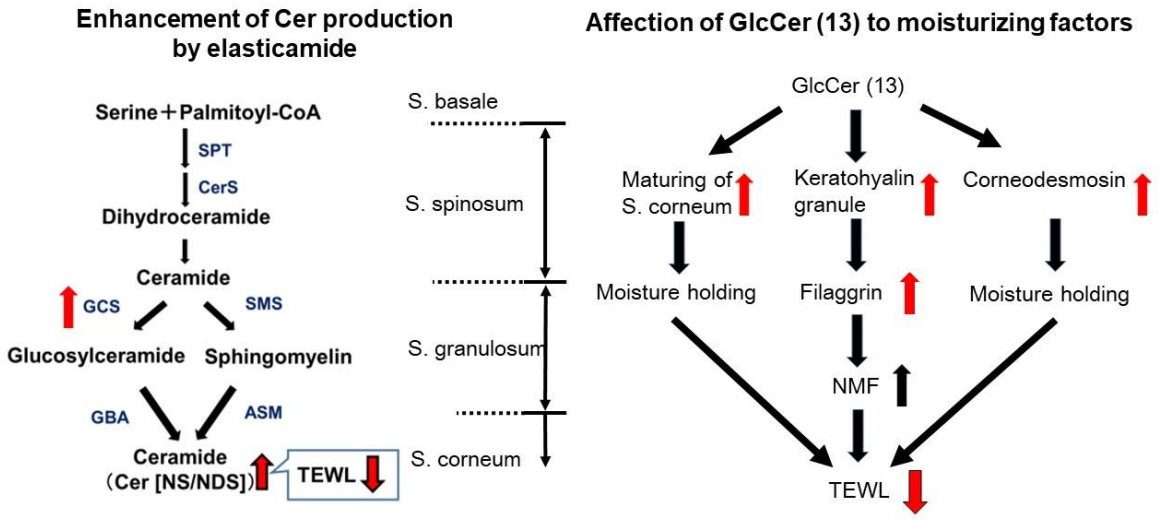

Figure 6. Possible moisturizing mechanism of elasticamide (**14**) and GlcCer (**13**)

Inhibitory effects of GlcCer (1-11) and elasticamide (14) on melanogenesis in B16 melanoma cells

Table 1 shows the inhibitory effects and cytotoxicity of GlcCer (**1**-**11**) and elasticamide (**14**) in B16 melanoma cells. GlcCer[d18:2(4*E*,8*Z*)/18:0] (**1**) significantly suppressed melanogenesis at 1 to 10 μg/mL without cytotoxicity. GlcCer[d18:2(4*E*,8*Z*)/20:0] (**3**) also inhibited melanogenesis from 1 to 10 μg/mL. However, **3** exhibited cytotoxicity at a concentration >3 μg/mL. The IC_{50} values of **1** and **3** on melanogenesis were less than 10 μg/mL. GlcCer[d18:2(4*E*,8*E*)/20:0] (**4**) and GlcCer[d18:1(4*E*)/20:0] (**6**) significantly suppressed melanogenesis at 10 μg/mL. GlcCer (**9**-**11**) with very long fatty acid lengths affected cell survival, but not melanogenesis. Other GlcCer, including GlcCer[d18:2(4*E*,8*Z*)/16:0] and GlcCer[d18:2(4*E*,8*E*)/16:0], did not have any effects on melanogenesis. Elasticamide (**14**), a Cer[t18:0/24:0], strongly suppressed melanogenesis at concentrations >1 μg/mL with an IC_{50} value of 3.9 μM.

Table 1. Inhibitory activities of GlcCer (**1**-**11**) and elasticamide (**14**) against theophylline-stimulated melanogenesis and cell viability in B16 melanoma cells

Sample Conc. (μg/mL)	1	3	10	IC_{50} (μM)
GlcCer[d18:2(4*E*, 8*Z*)/16:0]	8.2±3.7 (96.4±1.0)	4.3±5.3 (97.5±0.8)	4.3±3.0 (95.9±0.9)	
GlcCer[d18:2(4*E*, 8*E*)/16:0]	8.1±3.3 (96.8±0.6)	10.8±3.4 (96.7±0.9)	5.8±3.3 (95.2±0.4)	
GlcCer[d18:2(4*E*,8*Z*)/18:0] (**1**)	29.7±1.3** (90.6±5.8)	45.3±3.4** (84.1±1.1)	69.8±2.9** (95.8±2.8)	6.6
GlcCer[t18:1(8*Z*)/20:0] (**2**)	5.3±6.7 (85.8±3.6)	15.9±4.7 (79.7±10.4)	-12.9±8.2 (78.1±4.9)	
GlcCer[d18:2(4*E*,8*Z*)/20:0] (**3**)	22.9±2.4** (91.6±5.4)	45.8±2.1** (70.3±3.2**)	63.8±1.4** (63.2±2.9**)	5.9
GlcCer[d18:2(4*E*,8*E*)/20:0] (**4**)	36.1±1.1** (88.6±3.3)	44.0±2.5** (88.2±5.6)	45.6±1.3** (91.0±2.1)	>10
GlcCer[t18:1(8*Z*)/22:0] (**5**)	-5.9±4.2 (115.1±8.3)	1.6±5.1 (86.4±5.1)	11.2±1.5 (81.9±6.3)	
GlcCer[d18:1(4*E*)/20:0] (**6**)	17.3±1.5 (97.7±1.5)	4.8±2.5 (97.4±0.8)	32.7±4.8** (95.8±0.2*)	>10
GlcCer[d18:2(4*E*,8*Z*)/22:0] (**7**)	7.8±8.1 (81.5±4.1)	-22.7±5.2 (97.1±7.9)	0.0±7.8 (100.6±7.0)	
GlcCer[d18:2(4*E*,8*E*)/22:0] (**8**)	13.7±5.4 (94.6±2.6*)	22.5±3.8* (97.3±1.4)	0.0±2.6 (95.7±0.9)	
GlcCer[t18:1(8*Z*)/24:0] (**9**)	18.8±2.1* (49.6±2.7**)	26.6±4.5** (51.3±1.9**)	21.0±2.8* (63.1±2.9**)	
GlcCer[d18:2(4*E*,8*Z*)/24:0] (**10**)	8.2±3.7 (62.4±5.5**)	4.3±5.3 (73.6±5.5**)	4.3±3.0 (101.3±4.3)	
GlcCer[d18:2(4*E*,8*E*)/24:0] (**11**)	5.7±1.5 (48.5±0.5**)	2.5±0.9 (68.8±1.1**)	8.5±0.9 (84.1±1.3**)	

Elasticamide, Cer[t18:0/24:0](**14**)	21.7±5.2** (98.1±6.0)	46.4±2.7** (100.8±7.2)	63.4±2.7** (86.3±6.7)	3.9
Sample Conc. (μM)	100	300	1000	IC_{50} (μM)
Kojic acid	25.9±3.2** (96.5±0.4)	50.4±0.7** (99.5±1.8)	74.4±1.1** (87.4±3.4*)	294.7

Cytotoxicity was indicated in parenthesis. Each value represents the means ± S.E. (N=4). Asterisks denote significant differences from the control group, *p<0.05, ** p<0.01.

Effects of GlcCer (3) and elasticamide (14) on the mRNA expression of melanogenesis-related enzymes, tyrosinase inhibitory activity, and ATP levels in B16 melanoma cells

We investigated whether **3** and **14** down-regulated the mRNA expression of melanogenic molecules in order to elucidate their mechanisms of action. As shown in Figure 7A, **14** significantly down-regulated the mRNA expression of TYRP1, whereas **3** did not. Therefore, the suppression of TYRP1 was involved in the anti-melanogenic effects of **14**. Neither **3** nor **14** affected the expression of MITF or TYR. Figure 7B shows the effects of **3** and **14** on tyrosinase activity derived from B16 melanoma cells. Kojic acid dose-dependently inhibited tyrosinase activity (IC_{50}:3.6 μM), whereas **3** and **14** did not. Moreover, **14** and emodin suppressed ATP levels, whereas **3** did not. Therefore, the anti-melanogenic effects of **3** were attributed to reductions in TYRP1 expression and ATP levels, and not to the inhibition of tyrosinase activity.

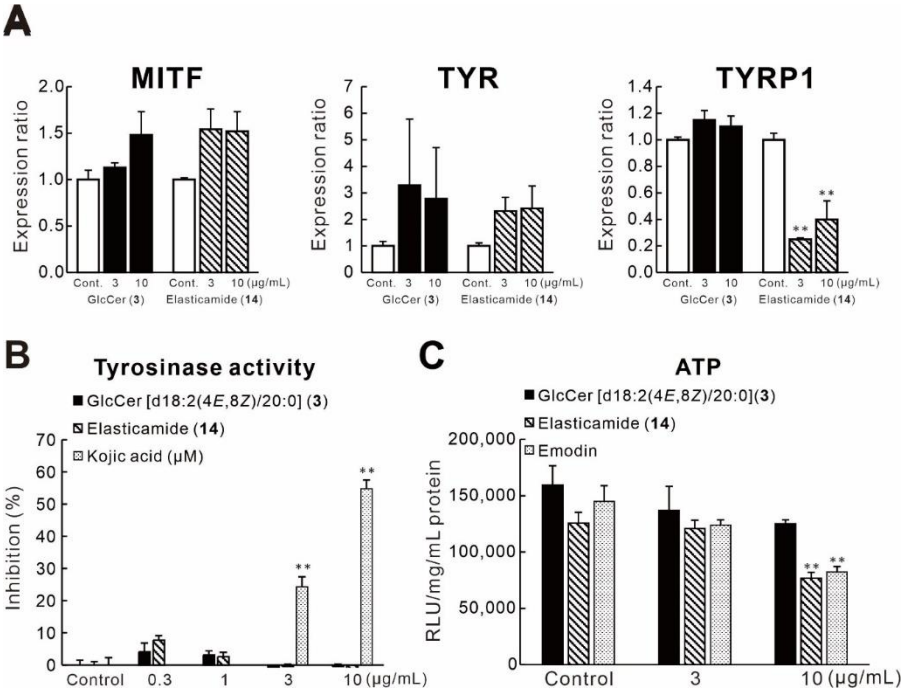

Figure 7. Effects of GlcCer[d18:2(4*E*,8*Z*)/20:0] (**3**) and elasticamide (**14**) on the mRNA expression of melanogenic factors (A), tyrosinase activity (B), and ATP levels in B16 melanoma cells (C).

A) B16 melanoma cells were treated with **3** or **14** for 72 hr. Each value was corrected by the mRNA expression level of β-actin and shown as a relative value to the control. B) Crude tyrosinase was extracted from B16 melanoma cells treated with theophylline for 72 h. Enzyme solutions were incubated with L-DOPA and test samples for 90 min. C) Cells were treated with **3** or **14** for 24 h. Data are expressed as the mean ± S.E. (n = 4). Asterisks denote significant differences from the control at $^{**}P<0.01$.

Effects of GlcCer (3) and elasticamide (14) on melanogenesis in 3D-cultured melanocytes and the mRNA expression of melanogenesis-related enzymes, tyrosinase inhibitory activity, and ATP levels in NHEMs

Figure 8A shows the anti-melanogenic effects of **3** and **14** on the content of melanin in MEL-300-A. Elasticamide (**14**) significantly decreased the content of melanin, whereas **3** did not. Observations of the upper side of the culture system and a microscopic analysis of Fontana-Masson staining showed that **14** suppressed melanin production in the basal layer. We also examined the effects of **3** and **14** on the mRNA expression of melanogenesis-related enzymes and cell viability in NHEMs. As shown in Figure 8B, **14** decreased the mRNA expression of TYRP1, similar to B16 melanoma cells, whereas **3** did not significantly affect the mRNA expression of any of the enzymes analyzed. Cell viability was not affected by **3** or **14**. UV-irradiated primary human keratinocytes and melanocytes release ATP, and increases in ATP levels activate tyrosinase and MITF. However, the results obtained showed that **3** and **14** did not reduce ATP levels.

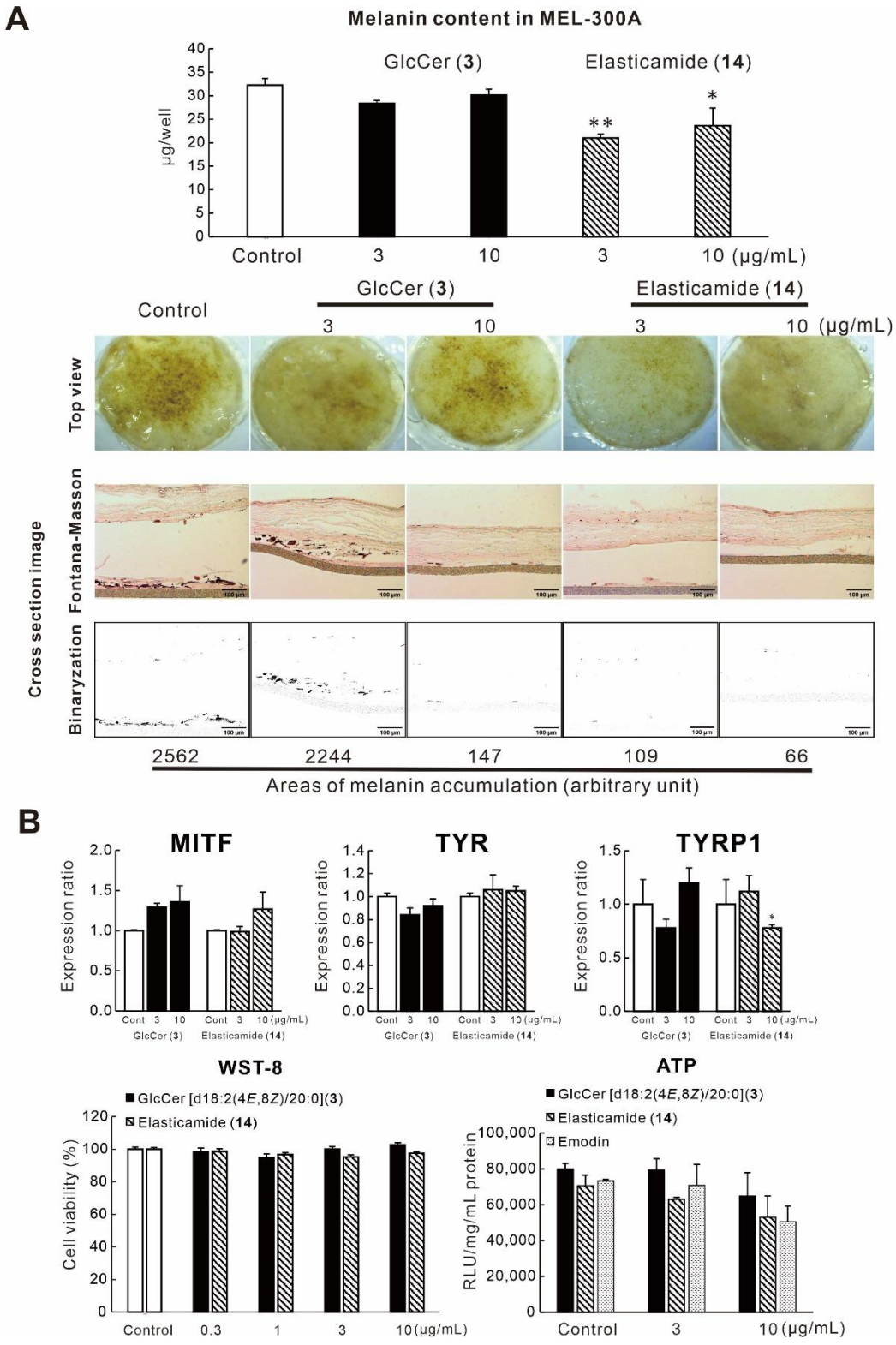

Figure 8. Effects of GlcCer[d18:2(4*E*,8*Z*)/20:0] (**3**) and elasticamide (**14**) on melanogenesis in MEL-300-A.

A) MEL-300-A were treated with **3** or **14** for 19 days. Data are expressed as the mean ± S.E. (n=4). Asterisks denote significant differences from the control at **P*<0.05 and ***P*<0.01. Middle microscopic images were taken from the upper side of the culture system. Pigmentation in microscopic cross-sectional images was assessed by Fontana-Masson staining. B) NHEMs were treated with **3** or **14** for 72 h in mRNA experiments, for 48 h in the WST-8 assay, and 24 h in the ATP analysis. Each value was corrected by the mRNA expression level of GAPDH and shown as a relative value to the control. Data are expressed as the mean ± S.E. (n=4). An asterisk denotes significant differences from the control at **P*<0.05.

Effects of Oryza ceramide containing GlcCer (3) and elasticamide (14) on UV-B-induced human skin pigmentation

After the 8-week intervention with Oryza ceramide®, skin pigmentation observed 7 days after UV irradiation (1288 and 1546 mJ/cm^2·S) was suppressed by Oryza ceramide® at 8 weeks (Figure 9).

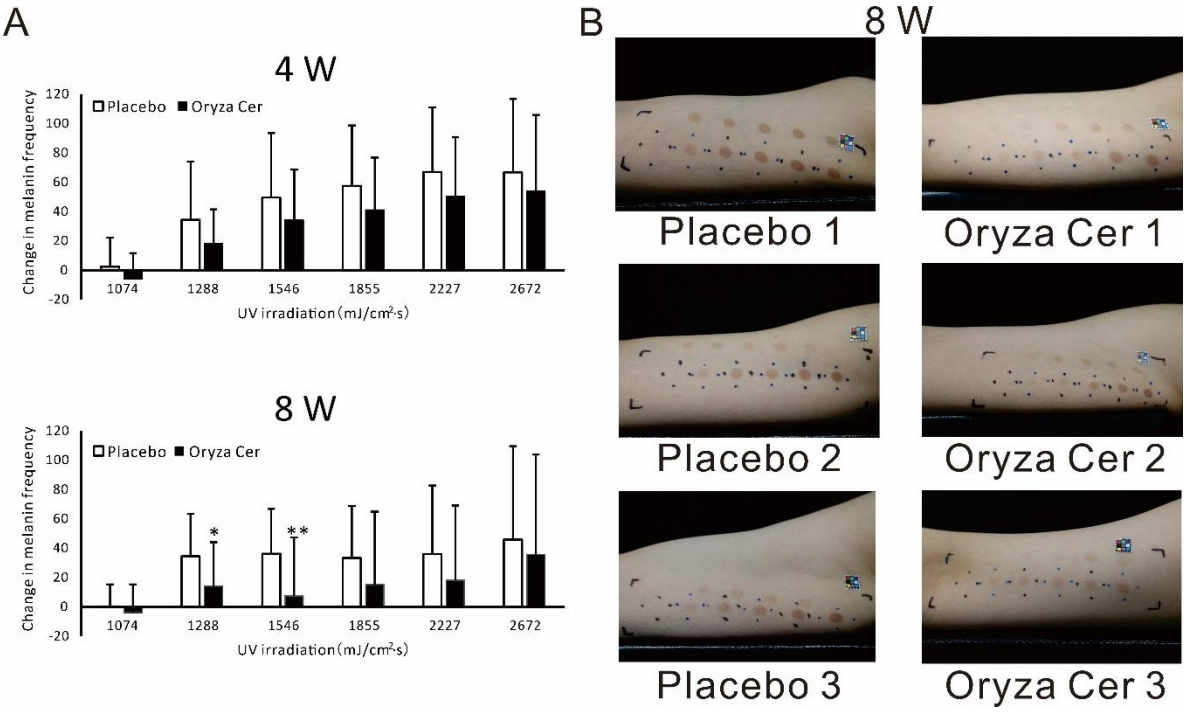

Figure 9. Effects of Oryza Ceramide®-PCD on skin pigmentation induced by UV irradiation.

A) Changes in melanin frequency in UV-irradiated skin. Each value represents the mean and S.D. (n=24). B) The arm skin of 3 typical subjects in each group exposed to UV irradiation after 8 weeks. Asterisks denote significant differences from the control at *P<0.05 and **P<0.01.

Discussion. In this study we have isolated 13 GlcCer and a Cer from rice and evaluated the moisturizing and anti-melanogenic effects. For evaluation of the moisturizing effect, we used RHEK and measured TEWL which is also a parameter of barrier function. The structure-activity relationships were clearly demonstrated in terms of GlcCer. Moisturizing effects were only found in GlcCer[d18:2(4*E*,8*Z*)] (**1**,**3**,**7**,**10**,**13**) and GlcCer[t18:1(8*Z*)] (**2**,**5**,**9**,**12**) and GlcCer[d18:2(4*E*,8*E*)] (**4**,**11**) exhibited no significant effects. Active GlcCer (**1**,**3**,**7**,**10**,**13**) showed fatty acid length-dependent epidermal hydrating effects. Thus, longer fatty acids appeared to enhance hydrating effects of GlcCer. The moisture retention effects of **10** and **13** with more than C24 fatty acids emmerged in earlier stage of culture (Days 1 and 3). In contrast, elasticamide (**14**), a Cer[AP], showed strong moisturizing effect in later stage of culture (Days 5 and 7). Thus, moisturizing mechanisms of **13** and **14** appeared to differ.

We then determined Cer contents in RHEK treated with GlcCer and **14** to find the possible moisturizing mechanism. As a result, **14** increased Cer contents in RHEK specifically Cer [NS/NDS], while GlcCer (**1**-**5**, **7**, **9**-**13**) have no effect on Cer content. Interestingly, **14** did not increase Cer[AP] in spite of possessing a same structure. As Cer [NS/NDS] are major lipids in SC Cer [17], we evaluated the mRNA and protein expressions of enzymes involved in *de novo* Cer synthesizing pathway [18] on RHEK treated with **14**. As a result, **14** enhanced GlcCer synthase (GCS) expression, which facilitates change from Cer to GlcCer in S. granulosum [19]. Therefore **14** was found to exhibit moisturizing effect through production of Cer based on GCS expression.

In contrast, as **13** did not change Cer contents in spite of dose-dependent TEWL reduction, we performed electron microscopic analysis to find the hint of moisturizing mechanism of **13**. As a result, denser SC image of **13**-treated RHEK suggested that the structure could hold more moisture. Moreover, the numbers and areas of keratohyalin granules were increased by **13**. Keratohyalin granules primary exist in S. granulosum and contribute to cross-linking of keratin filaments which creates the tight barrier in the epidermis [20]. The granules also produce

filaggrin which is precursor of natural moisturizing factor (NMF) [20]. Actually, western blot analysis provided us the evidence of increase in filaggrin expression. Therefore, **13** was suggest to exhibit moisturizing effect by increase in filaggrin which provides NMF to SC. In addition microscopic images of immunostaining and western blot analysis showed enhancement of corneodesmosin expression. Corneodesmosin, a cell adhesion molecule, exists in corneodesmosome cores and contributes to tight SC structure [21]. Thus, the effect on corneodesmosin could be another moisturizing mechanism of **13**.

To sum up the moisturizing effects, only GlcCer with 4*E*,8*Z* structure possessed moisturizing effects. Longer fatty acid enhanced the effect. The moisturizing mechanism involved enhancement of tight SC structure and NMF production. On the other hand, the main moisturizing mechanism of elasticamide (**14**) was SC Cer [NS/NDS] production through GCS expression.

In terms of anti-melanogenic effect in B16 melanoma cells, 4*E*,8*Z* type GlcCer (**1**, **3**) with C18 and C20 fatty acids strongly suppressed melanogenesis induced by theophylline without or less cytotoxicity. Regarding comparison of the activity and structures of similar GlcCer, in contrast to GlcCer (**3**), 4*E*,8*E* type GlcCer (**4**) has less anti-melanogenic activity. The result suggested that *cis* type configuration at C7-C8 position is necessary to exhibit anti-melanogenic activity of GlcCer[d18:2/18:0] and GlcCer[18:2/20:0]. However, GlcCer[d18:2(4*E*,8*Z*)] with different length of fatty acids such as C16, C22 (**7**), and C24 (**10**) fatty acids have no anti-melanogenic activity. The detailed structure-activity relationship of anti-melanogenic activity was not able to be clarified yet, however at least, major GlcCer (**3**) was found to possess strongest anti-melanogenic activity in rice GlcCer (**1**-**13**). Besides, GlcCer with longer free fatty acid appeared to show stronger cytotoxicity against B16 melanoma cells. Cytotoxicity of GlcCer (**9**-**11**) with C24 fatty acids to B16 melanoma were stronger than the other GlcCer (**1**-**8**) with C16 to C22 fatty acids.

In contrast to GlcCer, elasticamide (**14**), a free ceramide with C24 fatty acid, exhibited strongest anti-melanogenic effect among samples we tested in B16 melanoma cells with IC_{50} of 3.9 μM. Differing from GlcCer (**9**-**11**), **14** with C24 fatty acid has no double bond in the sphingoid base (t18:0). Thus, existence of glucose or double bond appeared to diminish anti-melanogenic activity. Moreover, the structure of **14** is same as ceramide [AP] which is

existing in skin SC. Therefore, elasticamide was suggested to play suppressive role on innate skin melanin production.

Then we evaluated anti-melanogenic mechanisms of **3** and **14** in B16 melanoma cells. Elasticamide (**14**) but not **3** suppressed mRNA expression of tyrosinase related protein (TYRP) at 3 and 10 μg/mL. Both **3** and **14** affected no influence to tyrosinase activity derived from B16 melanoma cells. This result corresponds to the previous report [11] reporting that yeast and bovine brain cerebrosides (perhaps free ceramide mixture) did not suppress tyrosinase activity derived from B16 melanoma. Although same report [11] reported that both cerebrosides suppressed tyrosinase expression in protein level, our data of tyrosinase mRNA (TYR) was not suppressed by both **3** and **14**. Thus the difference of ceramide structure might be involved in the difference of the results and the other types of free ceramides could suppress tyrosinase expression.

On the other hand, **14** at 10 μg/mL significantly suppressed ATP concentration in B16 melanoma. Hence, anti-melanogenic mechanism of **14** is considered to involve the suppression of ATP production which is required to promote energy for cell survive. In B16 melanoma cells, ATP is needed for proliferation through P2X7 receptor [22] and melanogenesis [23]. However inhibitory mechanism of **3** was not clarified yet. To guess the reason why **3** did not affect to expression of melanogenic factors in B16 melanoma, we referred anti-melanogenic effects of several fatty acids. Aida et al [24] have described that C20 saturated fatty acid (icosaenoic acid) which is a partial structure of **3** did not suppressed melanogenesis in B16 melanoma. Instead, suppression of melanogenesis was observed by the treatments of non-saturated fatty acids such as palmitoleic acid, oleic acid and linoleic acid. These fatty acids also suppressed tyrosinase activity. Therefore, free fatty acid (icosaenoic acid) in **3** was not seemed to be involved in anti-melanogenic effect of **3**.

Regarding the effects of sphingoids on MITF expression in melanoma, C2 ceramide have been reported to suppress MITF expression [25]. As both **3** and **14** did not suppress MITF mRNA expression in our study, thus, the inhibitory mechanisms of rice-derived GlcCer (**3**) and **14** might be MITF independent differing from simple sphingolipids or ceramides. On α-MSH-induced melanogenesis in normal melanocytes, elasticamide **14** suppressed melanogenesis and TYRP1 mRNA expression as well as on B16 melanoma cells. However, **14** did not affect to ATP levels in normal melanocytes in contrast to in B16 cells. Thus, **14**

was found not to affect to ATP level in normal melanocytes and suppress melanoma ATP. The main anti-melanogenic mechanism of **14** appeared to be TYRP1 suppression. On the other hand, GlcCer (**3**) did not suppress melanogenesis, melanogenic protein expression and ATP level in normal melanocytes. In this study we chose α-MSH to induce melanogenesis, because co-stimulation with ET-1 and SCF [26] could not induce melanogenesis. Thus at least, **14** was found to have no anti-melanogenic activity on α-MSH-induced melanogenesis. However, it may affect to melanogenesis induced by other melanogenic agents such as ET-1, SCF, and ACTH [27] and further investigation is required to clarify the anti-melanogenic mechanism of **3**.

On clinical study of orally ingested Oryza ceramide® containing both rice GlcCer (**1**-**13**) and **14**, it suppressed UV-induced skin tanning (melanin pigmentation). It only suppressed skin tanning and not suppressed erythema. As there are no reports of antioxidant activities of GlcCer and Cer like xanthophyls and polyphenols, it is reasonable that Oryza ceramide® did not exhibit UV-induced inflammation caused by reactive oxidant species (ROS). On the suppression of UV-induced tanning by Oryza ceramide®, GlcCer and **14** are appeared to be involved by suppressing melanin accumulation.

Conclusion. We have demonstrated that GlcCer [d18:2(4*E*,8*Z*)] exhibited fatty acid length-dependent moisturizing effect in a epidermis model. The mechanism seems to involve enhancement of density of SC and filaggrin and corneodesmosin expressions. Whereas, elasticamide showed the moisturizing effect by accelerating SC Cer [NS/NDS] production through GCS. In terms of anti-melanogenic effects, GlcCer [d18:2(4*E*,8*Z*)] with C18 or C20 fatty acid and elasticamide suppressed melanin production in melanoma cells. The inhibitory mechanism of GlcCer was not clarified; however, the effect of elasticamide involved suppression of TYRP1 and ATP expression. This study is the first report which examined moisturizing and anti-melanogenic effects of Cer species in single molecule level.

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Conflict of Interest Statement. NONE.

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