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Soyasaponin-I Attenuates Melanogenesis through Activation of ERK and Suppression of PKA/CREB Signaling Pathways

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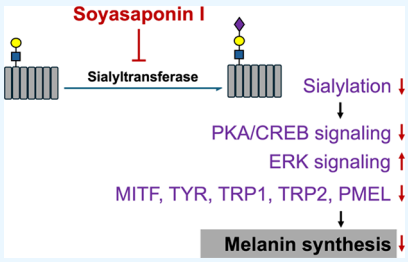
ABSTRACT: Soyasaponin-I (SS-I), a sialyltransferase inhibitor naturally found in soybeans, has antioxidant, anticarcinogenic, and hepatoprotective properties. In this study, we explored the possibility to use SS-I as an antimelanogenic agent for the treatment of skin hyperpigmentation disorders. When melanoma cell lines, MNT-1 and B16F10, were treated with SS-I, significant suppression of both α-2,3 and α-2,6 sialylations was observed by using lectin fluorescence staining with sialic acid-binding lectins—*Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* lectin-II (MAL-II). SS-I significantly attenuated the α-MSH-induced melanogenesis of MNT-1 and B16F10 cells without a cytotoxic effect. SS-I could activate ERK and suppress the PKA/CREB signaling pathways of melanoma cells. Moreover, SS-I treatment caused significant downregulation of the expression of melanosome-related proteins; tyrosinase-related protein 1 (TRP1), TRP2, and premelanosome protein (PMEL) and the melanogenic-related transcription factor microphthalmia-associated transcription factor (MITF). Consequently, the expression of tyrosinase—the key enzyme regulating melanin production—was significantly suppressed after SS-I treatment. These results suggest the role of sialylation in melanogenesis and the possibility of using SS-I as an alternative antimelanogenic agent. In conclusion, we have demonstrated the antimelanogenic effect of SS-I, an active compound produced in soybeans. SS-I can be an antimelanogenic agent in cosmetic products for the treatment of hyperpigmentation disorders.



1. INTRODUCTION

Sialylation, the post-translational modification of proteins and lipids by *N*-acetyl neuraminic acid or sialic acid, plays significant roles in both normal physiology and human diseases.[1,2] In cancers, aberrant sialylation was associated with tumor progression and therapeutic resistance. Therefore, targeting sialylation can be an alternative approach for cancer treatment.[3−6]

Skin hyperpigmentation disorder is caused by increasing melanogenesis, the cellular process to produce melanin pigments in melanocytes. The α-melanocyte-stimulating hormone is an important factor in inducing melanin production in the cells. The previous study by Diwakar et al. showed that melanin synthesis and transportation were controlled by sialylation.[7] Suppression of sialylation by specific inhibitors significantly affects the melanin synthesis and transportation,[7] suggesting the possibility of targeting sialylation as a treatment for skin hyperpigmentation disorders.

Soybean is a source of natural bioactive compounds used in many medical and biological aspects.[8,9] Soyasaponin-I (SS-I) is one of the saponin compounds found in soybeans, and it has various biological activities, such as anti-inflammation and anticancer.[10−14] As an anticancer agent, SS-I inhibits metastatic potentials of breast cancer and melanoma cell lines.[13,14] Also, SS-I induces autophagy in cancer cells.[12] SS-I is a potent inhibitor for sialylation by competitive inhibition with CMP-Neu5Ac on α-2,3-sialyltransferase I (ST3Gal1). Suppression of ST3Gal activity by SS-I leads to a decrease of cellular sialylation. The cell surface sialic acids are important for the adhesion and migration abilities of the cancer cells.[10,13,14] This evidence suggests the potential of using SS-I to target sialylation in cancer cells for cancer treatment.

In this study, we explored the antimelanogenic effect of SS-I on MNT-1 and B16F10 melanoma cells. The possibility of using SS-I for the treatment of skin hyperpigmentation disorders was evaluated. The molecular mechanism by which SS-I attenuates melanogenesis was demonstrated. The results of this study provided evidence of the use of SS-I, a natural active compound from soybean, for cosmetic purposes.

2. MATERIALS AND METHODS

This study was approved to be exempt from human ethics clearance by the Office of the Khon Kaen University Ethics Committee in Human Research based on the Declaration of

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Helsinki and the ICH Good Clinical Practice Guidelines (HE671766).

2.1. Cell Lines and Cell Culture. MNT-1 (human melanoma cell line) and B16F10 (mouse melanoma cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Brooklyn, New York) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Brooklyn, New York) and 1% antibiotic−antimycotic (Gibco, Brooklyn, New York) at 37 °C in a 5% CO_2 incubator. At 80−90% cell confluency, the cells were harvested using 0.05% (w/v) trypsin-EDTA (Gibco, Brooklyn, New York) and processed to the particular assays.

2.2. Melanin Assay. Melanin production was measured using the protocol modified from the previous study.[15] MNT-1 and B16F10 cells were seeded and cultured in a six-well plate for 24 hours (h) and then treated with 1, 5, and 10 μM SS-I (Sigma-Aldrich, St. Louis, Missouri) for 48 h, followed by 12.5 nM α-melanocyte-stimulating hormone (α-MSH) for additional 48 h. Cells treated with 0.5% ethanol, a comparable ethanol concentration to the SS-I treatment, were used as a control. The cells treated with 500 μM kojic acid (Sigma-Aldrich, St.Louis, Missouri) were used as an antimelanogenic control. After treatment, the cells were harvested with 0.05% trypsin−EDTA and washed with PBS. To determine melanin contents, approximately 100,000 cells from each condition were solubilized with 100 μL of 2 M NaOH in 10% DMSO for 30 min (min) in boiled water (100 °C). The absorbance was measured at 450 nm. The melanin content was compared with that of the control ethanol-treated cells and expressed as % of the control. The experiment was repeated at least three times; the data from a representative experiment was presented.

2.3. Cell Viability Assay. Effect of SS-I on the cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MNT-1 and B16F10 cells were plated in the 96-well plate overnight and were treated with various concentrations (0−10 μM) of SS-I (Sigma-Aldrich, St. Louis, Missouri). After 72 h of culture, 10 μL of 5 mg/mL MTT (Invitrogen, Carlsbad, California) was added and incubated for 3 h, and the formazan crystal was solubilized by isopropanol. The absorbance was measured at 540 nm. Cells treated with 0.5% ethanol instead of SS-I were used as a control. The relative cell viability was calculated and presented as % of the control. The experiment was performed in five replicates and repeated three times, and the data shown here were from a representative experiment.

2.4. Immunofluorescent Staining. Lectin-cytofluorescence staining was used to determine sialylation in experimental cell lines. After treatments with SS-I for 72 h, the cells were washed twice with ice-cold PBS and fixed with methanol for 30 min. Nonspecific reactivity was blocked by incubating the cells with 3% bovine serum albumin (BSA) in PBS for 30 min. Then, the cells were incubated overnight at 4 °C with 80 μg/mL of biotinylated *Maackia amurensis* lectin-II (MAL-II, Vector Laboratories, Burlingame, California) for α2,3-sialylated glycans and 20 μg/mL biotinylated *Sambucus nigra* agglutinin (SNA, Vector Laboratories; Burlingame, California) for α2,6-sialylated glycans. After being washed with PBS, the cells were incubated with 1:500 Alexa488-conjugated streptavidin (Invitrogen, Carlsbad, California) in PBS at room temperature for 40 min. The nuclei were counterstained using 1:10,000 Hoechst33342 (Invitrogen, Carlsbad, California), and the signal was observed under a Zeiss LSM 800 Confocal Laser Scanning Microscope (Zeiss, Oberkochen, Germany). The fluorescent signal was quantitated using ImageJ software.[16]

2.5. Western Blotting. MNT-1 cells were cultured in a six-well plate for 24 h and then treated with 10 μM SS-I for 48 h, followed by 12.5 nM α-MSH for 48 h. The cells treated with 0.5% ethanol were used as a control. After washing with PBS, the cells were lysed with cell lysis buffer (8 M urea, 2 M thiourea, 150 mM NaCl, 50 mM Tris−HCl pH 7.4) containing protease inhibitors and phosphatase inhibitors (Roche, Mannheim, Germany). The cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). Nonspecific reactivity was blocked by incubation with 5% skim milk in PBS for 1 h. The membranes were then probed with the desired concentration of specific primary antibodies (1:1000 anti-tyrosinase, 1:1000 anti-tyrosinase-related protein (TRP)-1, 1:1000 anti-TRP-2, 1:1000 anti-premelanosome protein (PMEL), 1:1000 anti-melanogenic-related transcription factor−microphthalmia-associated transcription factor (MITF), 1:1000 anti-Akt, 1:1000 anti-pAkt (S473), 1:1000 anti-Erk, 1:1000 anti-pErk (T202/Y204), 1:1000 anti-pPKA (T197), 1:1000 anti-PKA, 1:1000 anti-pCREB (S133), 1:1000 anti-CREB (Cell Signaling Technology, Danvers, Massachusetts), and 1:10,000 anti-β-actin (Sigma-Aldrich, St.Louis, Missouri). The membranes were probed using the ECL Prime Western Blotting Detection System (Amersham, Buckinghamshire, UK), and the chemiluminescent signal was detected using the ImageQuant LAS 4000 mini-image analyzer and ImageQuant TL analysis software (GE Healthcare, Buckinghamshire, UK). The experiments were repeated at least three times.

2.6. Tyrosinase Activity. The effects of SS-I on the tyrosinase activity was determined using mushroom tyrosinase (Sigma-Aldrich, St. Louis, Missouri) and human tyrosinase extracted from MNT-1 cells, as previously described with slight modifications.[17] In brief, approximately 100 μg of MNT-1 cell lysate (human tyrosinase) or 100 μg of mushroom tyosinase was mixed with 1, 5, and 10 μM of SS-I in 100 mM potassium phosphate buffer pH 6.8 (total volume 90 μL), followed by 10 μL of 50 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA) (Sigma-Aldrich, St. Louis, Missouri), and incubated at 37 °C for 30 min in the dark. The 0.5% ethanol was used instead of SS-I as a control. After the incubation, the absorbance was measured at 450 nm. The inhibitory effect of SS-I on each compound was compared to the control. Kojic acid (500 μM) was used as a tyrosinase inhibitor reference. The experiment was performed in triplicate and repeated at least twice; the data from a representative experiment was presented.

2.7. Statistical Analysis. The experimental data are shown as mean ± standard deviation (SD). The difference between groups was compared by Student's t test in GraphPad Prism 10.4.1 software (GraphPad, Inc., La Jolla, California); $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Soyasaponin-I Suppresses Sialylation of Melanoma Cell Lines, MNT-1 and B16F10. To determine the effect of SS-I on the sialylation of melanoma cell lines, MNT-1 and B16F10, the cells were treated with various concentrations of SS-I (0, 1, 5, and 10 μM) for 72 h. Then, the level of sialylated glycans was determined using lectin fluorescence staining with

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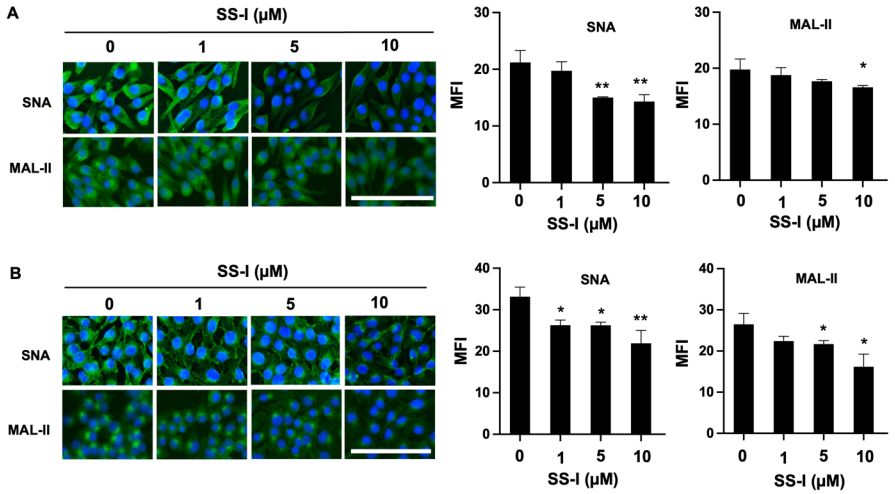

Figure 1. Effect of SS-I on sialylation of MNT-1 and B16F10 melanoma cell lines. The expression of the sialylated glycans was examined in (A) MNT-1 and (B) B16F10 cells using lectin fluorescence staining with MAL-II and SNA after treatment with SS-I for 72 h. Sialylated glycans were represented by Alexa 488 (green), and the nuclei were stained with Hoechst 33342 (blue). Scale bar: 50 μm.

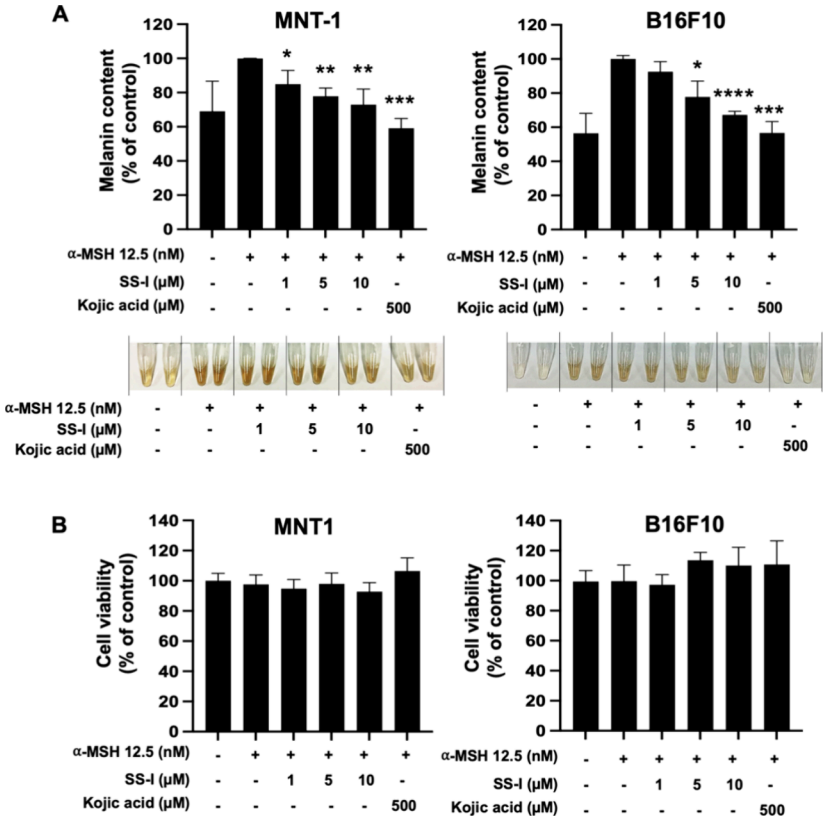

Figure 2. Effect of SS-I on melanin production and cell viability. (A) Melanin was dissolved using an alkaline−DMSO solution after SS-I and α-MSH treatments, and the absorbance was measured at 450 nm. The melanin content was calculated as % of the control. (B) The MTT assay was conducted to determine the cell viability 72 h after SS-I and kojic acid treatment. Asterisks indicate significant differences (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

MAL-II and SNA. The results indicated that SS-I suppressed the sialylation in the melanoma cell lines, as evidenced by the decrease of the expression of α2,3- and α2,6-sialylated glycans in MNT-1 and B16F10 cells in a dose-dependent manner (Figure 1A,B).

3.2. Soyasaponin-I Suppressed Melanin Production in Melanoma Cells. To determine the antimelanogenic effect of SS-I, MNT-I and B16F10 cells were treated with 1, 5, and 10 μM SS-I for 48 h, followed by 12.5 nM α-MSH for another 48 h. Melanin production was determined in comparison with 0.5% ethanol-treated cells as a negative control and 500 μM kojic acid-treated cells as a positive antimelanogenic control. The results showed that SS-I caused significant suppression of α-MSH-induced melanin production in MNT-I and B16F10 cells (Figure 2A) in a dose-dependent manner. Decreasing melanin production by SS-I treatment was correlated with the

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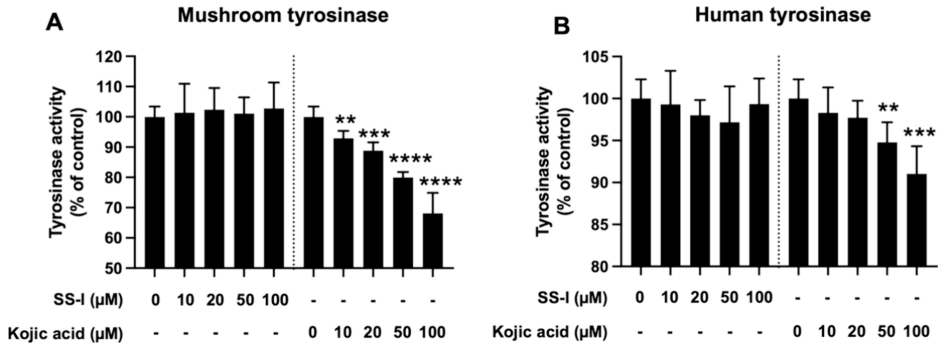

Figure 3. Effect of SS-I on tyrosinase activity. Effect of SS-I on the tyrosinase activity was examined using (A) mushroom tyrosinase and (B) human tyrosinase (extracted from MNT-1 melanoma cells). The enzymes were mixed with 0, 10, 20, 50, and 100 μM SS-I or kojic acid, using ethanol as a solvent control. The DOPA chrome was measured at 450 nm. The experiment was conducted in triplicate and repeated at least twice. Asterisks indicate significant differences (**P < 0.01; ***P < 0.001; ****P < 0.0001).

suppression of sialylated glycans (Supplementary Data). This information emphasizes the association between sialylation and melanin production.

To determine the cytotoxicity of SS-I, cell viability of MNT-1 and B16F10 cells was measured after treatment with SS-I and kojic acid for 72 h. The results showed that SS-I and kojic acid treatment did not affect the cell viability of the MNT-I and B16F10 cell lines (Figure 2B).

Moreover, we examined the effect of SS-I on the tyrosinase activity using tyrosinases from mushrooms and human cells (MNT-1). The results showed that SS-I (0−100 μM) does not affect the activity of both mushroom and human tyrosinases, suggesting that SS-I does not directly disrupt the function of the tyrosinase enzymes (Figure 3A,B). In contrast, the known tyrosinase inhibitor control, kojic acid, significantly inhibited both mushroom and human tyrosinases (Figure 3A,B).

3.3. Soyasaponin-I Suppressed Tyrosinase Expression and Melanogenesis via Activation of ERK and Suppression of PKA/CREB Signaling Pathways. Western blot analysis was employed to assess the effects of SS-I on the expression of TYR, melanosome-related proteins (TRP-1, TRP-2, PMEL), and the transcription factor MITF. The results showed that the expression of TYR, TRP-1, TRP-2, PMEL, and MITF was significantly suppressed after treatment with SS-I (Figure 4A). In terms of the signaling pathways of melanogenesis, the phosphorylation of ERK (pERK, T202/Y204) was significantly increased, while that of PKA (pPKA, T197) and CREB (pCREB, S133) was significantly decreased in comparison to the ethanol-treated control (Figure 4B). Phosphorylation of Akt (S473) remained unaffected by SS-I treatment. These findings suggested that SS-I regulates melanogenesis by activating ERK and suppressing PKA/CREB signaling pathways.

4. DISCUSSION

Melanin is a pigment produced from melanocytes and transported to keratinocytes; it protects the skin from the harmful effects of UV radiation. However, overproduction of melanin causes skin hyperpigmentation disorders, such as freckles, melasma, and age spots.[18] Recently, skin whitening or antimelanogenic products have become popular among Asian countries. Several natural and synthetic compounds have been used as an antimelanogenic ingredient in cosmetic products. The compounds, such as arbutin, thiamidol, and kojic acid, can suppress melanogenesis by different mechanisms, such as inhibition of tyrosinase (a rate-limiting enzyme of melanin

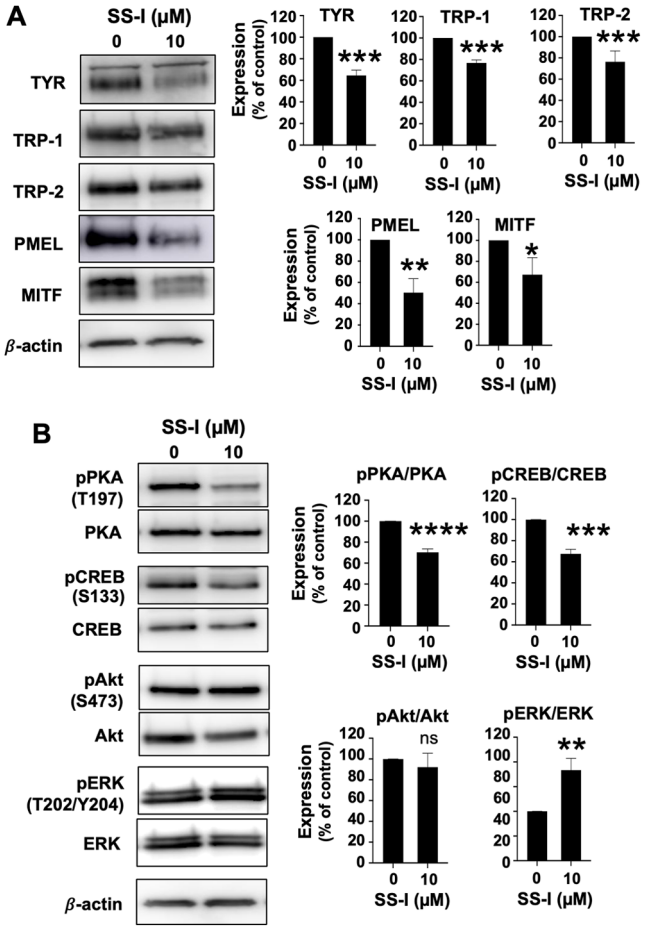

Figure 4. Effects of SS-I on the expression of melanogenesis-related proteins and cell signaling pathways. MNT-1 cells were treated with SS-I, compared with PBS, for 72 h. Western blot analysis was used to determine (A) the expression of TYR, TRP1, TRP2, PMEL, and MITF and (B) the melanogenesis-related signaling pathways: PKA/CREB, Akt, and ERK. Phosphorylated forms of each protein: pPKA (T197), pCREB (S133), pAKT (S473), and pERK (T202/Y204) were normalized by their total forms. β-Actin was used as an internal control. The graph was generated from an average value from three independent experiments. The blot images were from a representative experiment. Asterisks represented significant difference (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

production), suppression of melanosome formation, and melanin transfer.[7,18−20] Here, we demonstrated an antimelano-

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genic effect of SS-I, a sialyltransferase inhibitor naturally produced in soybeans. SS-I suppresses melanogenesis via the activation of ERK and suppression of PKA/CREB signaling pathways. Consequently, the tyrosinase and other melanin-related proteins (TRP-1, TRP-2, PMEL, and MITF) were suppressed after treatment with SS-I. Our data suggested the possible use of SS-I as an antimelanogenic agent for the treatment of hyperpigmentation disorders of the skin.

SS-I is a sialyltransferase inhibitor, suppressing ST3Gal1.[10,13,14] As SS-I is known to be a potent inhibitor of sialyltransferase, in our study, only SS-I was used to suppress the sialylation. Similar to ours, many studies used only SS-I to demonstrate the role of sialylation in particular models such as ovarian cancer, breast cancer, and melanoma.[13,14,21,22] Other sialyltransferase inhibitors, such as L-cytidine, 6-sialyl lactose, and 3-sialyl lactose, were previously used in the study of melanogenesis. Diwakar et al. demonstrated that inhibition of sialylation by sialyltransferase inhibitors (L-cytidine, 6-sialyl lactose, and 3-sialyl lactose) significantly reduced melanin production and transport.[7] In our study, SS-I could inhibit sialylation and melanin production in MNT-1 and B16F10 cells without a cytotoxic effect. To examine the toxicity of SS-I, we have examined the cytotoxic effect of SS-I up to 20 μM. Our data showed that SS-1 at 20 μM does not impact the cell viability of MNT-1 and B16F10 up to 96 h (Supplementary Data). In addition, we provide molecular insights into the mechanism of sialylation involved in melanogenesis. Our study demonstrated that sialylation regulates melanogenesis through the activation of ERK and the suppression of PKA/CREB signaling pathways. These pathways are involved in the regulation of downstream melanogenic-related proteins (MITF, TYP, TRP-1, TRP-2, and PMEL). SS-I treatment enhances the phosphorylation of ERK at Thr202 and Tyr204, which subsequently leads to the suppression of melanin production. This information aligns with previous studies showing that ERK phosphorylation at Thr202/Tyr204 increases MITF phosphorylation at Ser73, leading to its ubiquitination and subsequent proteasomal degradation.[23] To confirm the involvement of the ERK signaling pathway in melanogenesis, we treated MNT-1 cells with the MEK inhibitor (PD98059) and examined the melanin production. Our results agree with the previous studies that suppression of ERK by PD98059 significantly enhances melanin production (Supplementary Data).[24,25] In addition to activation of the ERK signaling pathway, the suppression of PKA/CREB also affects melanogenesis. Our data aligns with previous studies showing that the suppression of phosphorylation of PKA at Thr197 and CREB at Ser133 is associated with a decrease in melanin production.[26] The evidence presented in this study confirms the role of sialylation in melanogenesis and suggests the possible application of sialylation suppression in the treatment of hyperpigmentation disorders.

According to the tyrosinase activity assay, SS-I did not directly impact the activity of either mushroom or human tyrosinase. These results suggest that SS-I may not directly bind to or inhibit the function of tyrosinase. Instead, it may reduce the melanogenic effect of α-MSH. We speculated that sialylation might be important for the maturation of cell surface melanogenic-related glycoproteins such as melanocortin 1 receptor (MC1R). Perturbation of MC1R sialylation may influence its function and downstream signaling pathways related to melanin synthesis. Our experimental data support this hypothesis. However, further research on the sialylation of MC1R is needed to clarify this point. TYR was also modified by *N*-glycosylation, which is important for regulating its stability and function.[15,27] Terminal sialylation is a crucial step in the maturation of *N*-glycan. Defects in sialylation may contribute to the abnormal functions of glycoproteins. Therefore, the effect of SS-I on the sialylation of TYR cannot be ruled out. Since we have no direct evidence to demonstrate that sialylation of TYR is important for its function and stability yet, the effect of sialylation on TYR function requires further exploration.

In conclusion, we have demonstrated the molecular mechanisms through which sialylation regulates melanogenesis. The natural sialyltransferase inhibitor from soybean, SS-I, was identified as a potent antimelanogenic agent that could possibly be used for the treatment of hyperpigmentation disorders. We recommend SS-I as a candidate for use as an active ingredient in cosmetic products in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c01481.

Effect of soyasaponin-I on cell proliferation at 0−96 h; effect of SS-I on (PD98059) on the expression of sialylated glycans and melanin production; effect of the MEK inhibitor (PD98059) on cell proliferation and melanin production; and effect of kojic acid on cell proliferation (PDF)

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Notes

The authors declare no competing financial interest.

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