# Soyasaponin Ag inhibits α-MSH-induced melanogenesis in B16F10 melanoma cells via the downregulation of TRP-2

SEUNG HWAN YANG[1*], ARISTIDES M. TSATSAKIS[2*], GEORGE TZANAKAKIS[3],
HONG-SUK KIM[4], BAO LE[1], MARIA SIFAKI[2], DEMETRIOS A. SPANDIDOS[5],
CHIGEN TSUKAMOTO[6], KIRILL S. GOLOKHVAST[7], BORIS N. IZOTOV[8] and GYUHWA CHUNG[1]

[1]Department of Biotechnology, Chonnam National University, Yeosu, Chonnam 59626, Republic of Korea;
[2]Laboratory of Toxicology, Medical School; [3]Laboratory of Anatomy-Histology-Embryology, School of Medicine,
University of Crete, Heraklion 71003, Greece; [4]Durae Corporation, Research and Development Center, Gunpo, Gyeonggi 435-832,
Republic of Korea; [5]Laboratory of Virology, Medical School, University of Crete, Heraklion 71003, Greece; [6]Faculty of Agriculture,
Iwate University, Morioka, Iwate 020-8550, Japan; [7]Educational Scientific Center of Nanotechnology,
Engineering School, Far Eastern Federal University, Vladivostok 690950; [8]Department of Analytical Toxicology,
Pharmaceutical Chemistry and Pharmacognosy, Sechenov University, Moscow 119991, Russia

**Abstract.** Saponins, which are glycosylated, represent a diverse group of biologically functional products in plants. In the present study, we investigated the effects of soyasaponin Ag, a secondary metabolite extracted from soybean, on α-melanocyte-stimulating hormone (α-MSH)-induced melanin synthesis in B16F10 mouse melanoma cells and the underlying molecular mechanisms. To elucidate the mechanisms through which soyasaponin Ag inhibits melanin synthesis, we performed cellular tyrosinase activity assays and analyzed the expression of the melanogenesis-related genes, tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2. We demonstrated that soyasaponin Ag inhibited α-MSH-induced melanin synthesis in melanoma cells. Of note, soyasaponin Ag had no inhibitory effect on intracellular tyrosinase activity. However, soyasaponin Ag inhibited TRP-2 expression in a dose-dependent manner. Therefore, the depigmenting effect of soyasaponin Ag may be due to the inhibition of tyrosinase expression or the enhancement of tyrosinase degradation. Moreover, soyasaponin Ag did not exert any toxic on B16F10 mouse melanoma cells, suggesting that soyasaponin is a safe component for use in skin care cosmetic formulations that are used for skin whitening.

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*Correspondence to:* Professor Gyuhwa Chung, Department of Biotechnology, Chonnam National University, Yeosu, Chonnam 59626, Republic of Korea
E-mail: chung@chonnam.ac.kr

*Contributed equally

## Introduction

Melanin determines the color of human skin, hair and eyes as a final product of melanogenesis (1,2), and plays a protective role against sun exposure-associated ultraviolet radiation (3). This pigment is synthesized by a complex pathway in highly specialized cells of the epidermis known as melanocytes (4). Melanocytes are normally positioned at the basal layer of the epidermal part of the skin (5). Specifically, melanin is produced in cellular sub-compartments denominated as melanosomes and is transported to other cells of the epidermis, including keratinocytes and Langerhans cells (5). Importantly, the overproduction or abnormal distribution of melanin can lead to skin disorders and cosmetic complications, such as melisma and freckles (5). Melanin is synthesized by the three enzymes, tyrosinase, tyrosinase-related protein (TRP)-1 and DOPAchrome tautomerase (DCT) also known as TRP-2 (6). UV radiation-sensitized human epidermal keratinocytes produce factors which stimulate melanogenesis, including α-melanocyte-stimulating hormone (α-MSH) (7). Secreted α-MSH/ACTH binds to the melanocortin 1 receptor (MC1R) on melanocytes to activate the cAMP/PKA/CREB pathway, which subsequently increases the expression levels of tyrosinase, TRP-1 and TRP-2 (7,8). Tyrosinase converts L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA); L-DOPA can then be converted into DOPAquinone, which spontaneously undergoes cyclization and further rearrangement, yielding l-DOPAchrome (9). DCT/TRP-2 catalyzes the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). TRP-1 is important for tyrosinase stabilization and trafficking, thus ultimately facilitating tyrosinase activity (10). Two types of melanin are produced in mammalian cells, the reddish-yellow-colored pheomelanin and the dark-brown-colored eumelanin. Tyrosinase is required for the synthesis of pheomelanin and eumelanin, whereas TRP-1 and TRP-2 are required for the synthesis of only eumelanin (11,12).

Therefore, the downregulation of tyrosinase and related protein expression and/or activity have been proposed to be involved in the reduction of melanin production (13).

Soyasaponins are bioactive secondary plant metabolites in soybeans (*Glycine max*) and other legumes, such as lentils (*Lens culinaris*) and green peas (*Pisum sativum* L. cv. Azad) (14-16). Soyasaponins are oleanene-type triterpenoid saponins with polar sugar chains and a non-polar pentacyclic ring structure (17). These metabolites are generally divided into four main groups on the basis of their aglycone (soyasapogenol) structure, referred to as groups A, B, E and DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrone) (14). Group A soyasaponins contain bidesmosides with two sugar chains at the C-3 and C-22 position of soyasapogenol A. Previously, eight isomers of group A saponins, named Aa, Ab, Ac, Ad, Ae, Af, Ag and Ah, according to their elution order in reversed-phase high-performance liquid chromatography (HPLC), were isolated from soybeans and characterized in the study by Shiraiwa *et al* (15).

Soyasaponins have been shown to have a number of biological functions, including anti-obesity, anti-inflammatory and anti-carcinogenic effects, as well as hepatoprotective effects (14,18,19). However, to the best of our knowledge, no study to date has investigated the putative anti-melanogenic effects of soyasaponins. Therefore, in the present study, we investigated the anti-melanogenic effects of soyasaponin Ag on α-MSH-induced melanin synthesis in B16F10 melanoma cells.

## Materials and methods

*Chemicals and antibodies*. α-MSH, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), L-DOPA and arbutin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to tyrosinase (sc-73244), TRP-1 (sc-58438), TRP-2 (sc-74439) and microphthalmia-associated transcription factor (MITF; sc-52938) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

*Isolation of soyasaponin*. Soybean saponins Ag were purified by the method previously described in the study by Shiraiwa *et al* (15) with some modifications. The seed hypocotyls were extracted in a 10-fold volume (v/v) of 80% (v/v) aqueous methanol for 24 h at room temperature. The extracts were concentrated *in vacuo* to evaporate the solvents. The dried extracts were suspended in a mixture of 2-butanol, water and acetic acid (final concentration, 0.1%; equal to the 10-fold volume used for extraction) and followed by overnight incubation at room temperature. Following incubation, the upper (butanol) phase was collected, evaporated and dried under a vacuum. To prepare crude saponin solutions, the dried powders were dissolved in a small amount of preparative HPLC solvent [34% (v/v) acetonitrile, 6% (v/v) 2-propanol, 0.1% (v/v) acetic acid and 59.9% (v/v) water]. The crude saponin solutions were then filtered through 0.45-mm membrane filters and were analyzed repeatedly on a preparative reverse-phase HPLC system. The purity of saponin Ag measured by liquid chromatography-photodiode array-tandem mass spectrometry (LC-PDA-MS/MS) analysis was >95%, based on the total peak area monitored at 205 nm.

*LC-PDA-MS/MS analysis*. Analysis of the saponin components was carried out by LC-PDA-MS/MS with an analytical

reverse-phase column (Develosil C30-UG-5, 4.6x150 mm; Nomura Chemical Co., Ltd., Aichi, Japan) at 40°C. Solvent A (94% acetonitrile, 6% 2-propanol and 0.1% formic acid) and solvent B (94% water, 6% 2-propanol, and 0.1% formic acid) were used. Gradient elution from 10.64% (v/v) solvent A (10% acetonitrile) to 53.19% (v/v) solvent A (50% acetonitrile) was performed for 40 min (gradient increased by 1% acetonitrile per minute) at a flow rate of 0.15 ml/min (Prominence UFLP system; Shimadzu Corp., Kyoto, Japan). Then, the column was washed by increasing the concentration of solvent A to 100% (v/v) for 5 min; after which, the eluates composition returned to the initial state of 10.64% (v/v) solvent A for 15 min. The eluate from the column was monitored at 205 and 292 nm using a PDA detector, and MS and MS/MS data of the eluates were acquired with a tandem mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Inc., Waltham MA, USA) in positive-ion mode using electrospray ionization [ESI(+)]. Saponin contents were calculated from a standard curve on the basis of the peak area of purified saponin Ag monitored at 205 nm.

*Cell culture*. B16F10 cells, established to produce melanin and respond to α-MSH (20) were purchased from the American Type Culture Collection (ATCC; CRL-6475). Typically, the B16F10 cells were cultured in α-modified Eagle's minimal essential medium containing 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (10,000 unit/10,000 $\mu$g/ml) (Gibco, Grand Island, NY, USA) in a humidified atmosphere containing 5% $CO_2$ in air at 37°C.

*Cell viability assay*. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). The B16F10 cells were cultured in 96-well plates. After a 24-h incubation, the cells were treated with soyasaponin Ag (25-100 $\mu$M) and then incubated for 48 h in an atmosphere of 5% $CO_2$ at 37°C. After 48 h, MTT solution (5 mg/ml) was added to each well and the cells were incubated for 4 h at 37°C. The supernatants were removed, and dimethylsulphoxide (DMSO) was added to dissolve the formazan crystals. The absorbance of each well was estimated at 570 nm using an ELISA plate reader (Tecan, Mannedorf, Switzerland). The percentage of living cells was determined relative to the control group.

*Melanin content assay*. The B16F10 cells ($3.5x10^4$) were cultured in 6-well plates at 37°C in an atmosphere of 5% $CO_2$ for 24 h. After 24 h, the cells were treated with α-MSH (20 nM) and medium containing arbutin (Sigma-Aldrich; arbutin was diluted in 0.1 M phosphate buffer and used at 1 and 2 mM concentrations as a positive control) or soyasaponin Ag (25, 50 and 100 $\mu$M) for 48 h. After 48 h, the cells were washed with phosphate-buffered saline (PBS), and the cells were detached by incubation with trypsin-EDTA and centrifuged at 3,000 rpm for 5 min. The supernatant was then discarded and the cell pellet was solubilized in 1 N NaOH at 60°C for 2 h. To determine the melanin content, the absorbance was measured at 405 nm using an ELISA plate reader and then normalized to the total protein concentration.

*Cellular tyrosinase activity assay*. B16F10 cell seeding and treatment methods were the same as those used to measure
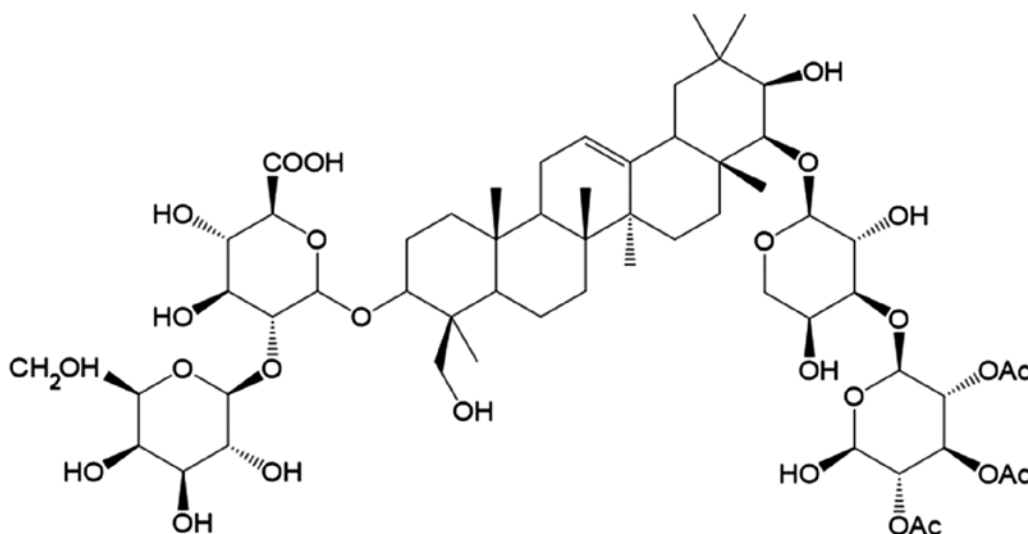
Figure 1. Chemical structure of soyasaponin Ag.

melanin contents. Briefly, the cells were cultured in 6-well plates, and after 24 h, the cells were treated with α-MSH (20 nM) and soyasaponin Ag-containing medium (25-100 µM) for 48 h. After treatment, the cells were washed twice with cold PBS and lysed with 100 µl of RIPA buffer containing 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF) for 30 min on ice. The lysates were centrifuged (13,000 rpm for 15 min), 20 µl of the supernatant was mixed with 100 µl of 4% N,N-dimethylformamide, 50 µl of 5 mM L-DOPA and 50 µl of 20.7 mM 3-methyl-2-benzothiazo-linone hydrazine (MBTH) in a 96-well plate and then incubated at 37°C for 30 min. To determine the tyrosinase activity, the absorbance was measured at an absorbance wavelength of 505 nm, and then normalized to the total protein concentration.

*Western blot analysis*. The B16F10 cells were treated with soyasaponin Ag (25-100 µM) for 24 h and then further treated with α-MSH (20 nM) and the soyasaponin sample for a further 24 h. After these treatments, the cells were collected and lysed with RIPA buffer containing 1X solution (50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) for 30 min on ice. The cell lysates were centrifuged (10,000 rpm for 10 min), and the protein concentrations were determined using the BCA protein assay. Proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked with blocking buffer (Thermo Fisher Scientific, Inc.) and then incubated with the appropriate antibodies. The membranes were washed with wash buffer and incubated with anti-mouse (sc-2371) or anti-rabbit (sc-2379) horseradish peroxidase-conju-gated secondary antibodies (Santa Cruz Biotechnology, Inc.) at 1:1,000 dilution for 1 h. The immunoreactive bands were enhanced using chemiluminescence reagents. The loading control was assessed using an anti-β-actin antibody.

*Statistical analysis*. All data were obtained in triplicate and are presented as the means ± standard deviation. Values were compared using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Effect of soyasaponin Ag on cell viability and melanin synthesis in α-MSH-stimulated B16F10 cells*. The chemical structure of saponin Ag is shown in Fig. 1. The first step in this study was to determine any potential cytotoxic effects of soyasaponin Ag in view of its potential utilization as a functional food or cosmetic agent. We performed cell viability assays with soyasaponin Ag to determine its *in vitro* cytotoxicity in murine B16F10 melanoma cells. More specifically, we utilized the MTT colorimetric assay for cell viability. In short, MTT is reduced to purple formazan crystals by the enzymatic activity of the mitochondria. Since for most living cells total mitochondria activity correlates with the number of viable cells, the assay is a simple and reliable method for determining potential cytotoxic effects (21). To determine the effect of soyasaponin Ag on B16F10 cell viability, the cells were treated with 25-100 µM of soyasaponin Ag for 48 h (Fig. 2A). No significant effect on cell viability was detected when using this concentration range of soyasaponin Ag. To determine the effect of soyasaponin Ag (25-100 µM) treatment on α-MSH-induced melanin synthesis, the melanin content of the B16F10 cells was evaluated photometrically. As shown in Fig. 2B, α-MSH markedly upregulated the B16F19 cell melanin content (P<0.05). Of ntoe, treatment with soyasaponin Ag at 50 and 100 µM significantly decreased the cellular melanin content of the α-MSH-stimulated B16F10 cells (P<0.01). In the control experiments, arbutin (positive control), a known melanogenic inhibitor (22) was shown to attenuate the melanin content of stimulated B16F10 cells (Fig. 2B).

*Effect of soyasaponin Ag on tyrosinase activity in α-MSH-stimulated B16F10 cells*. Tyrosinase is known to play a key role in melanin synthesis. Thus, in this study, to investigate the mechanisms underlying the inhibition of melanogen-esis, we measured tyrosinase activity in α-MSH-stimulated B16F10 cells (Fig. 3). B16F10 melanoma cells treated with up to 100 µM soyasaponin Ag did not exhibit any inhibition of tyrosinase activity. On the other hand, in the control experi-
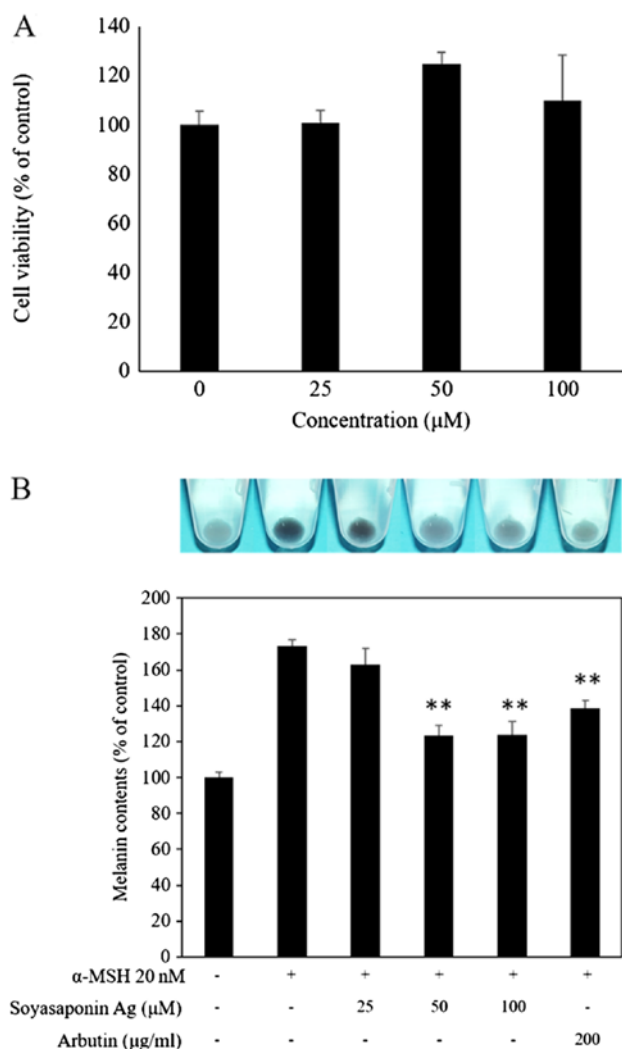
Figure 2. Effects of soyasaponin Ag on (A) cell viability and (B) melanin content in α-MSH-stimulated B16F10 cells. α-MSH, α-melanocyte-stimulating hormone. **P<0.01.



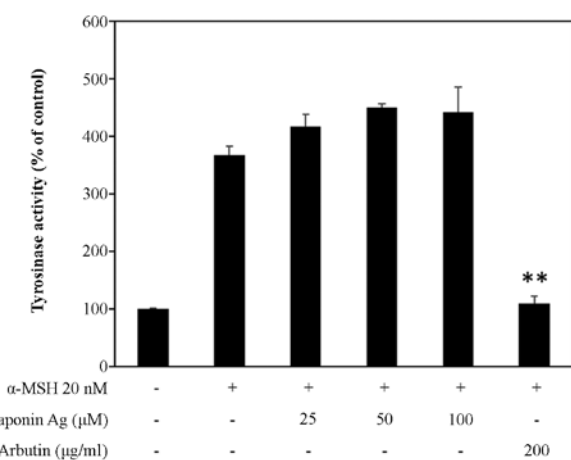Figure 3. Wffects of soyasaponin Ag on the cellular tyrosinase activity of α-MSH-stimulated B16F10 cells. α-MSH, α-melanocyte-stimulating hormone. **P<0.01.
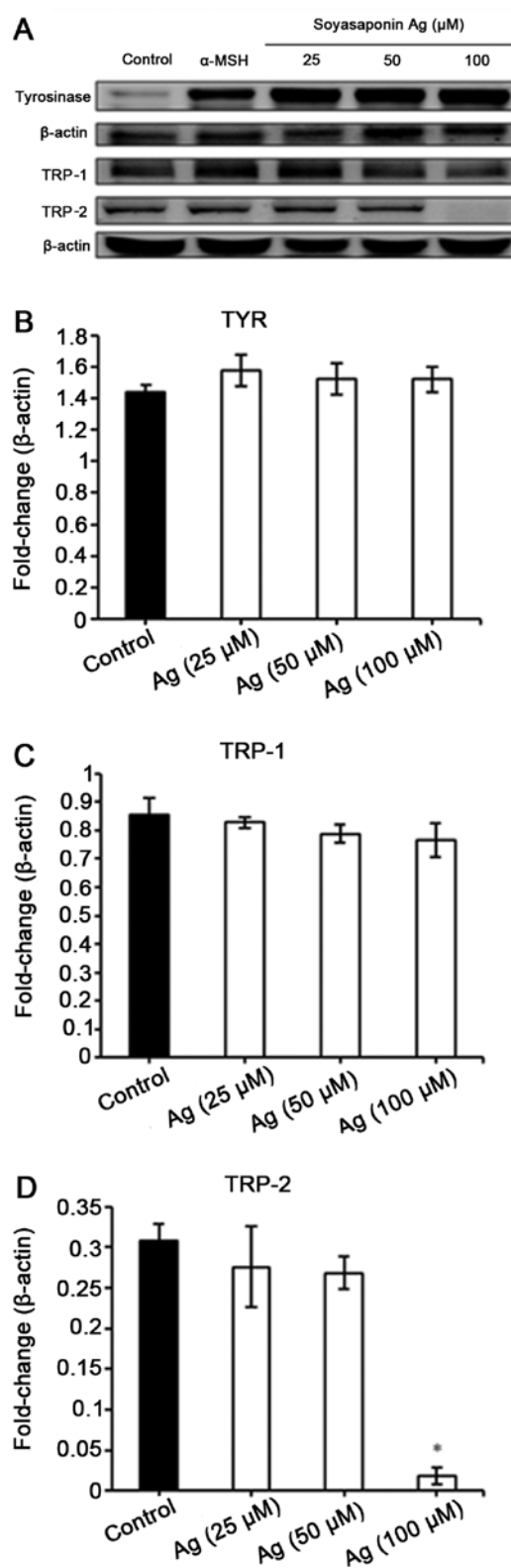


Figure 4. Effects of soyasaponin Ag on the protein levels of melanogenic factors in B16F10 cells. TYR, tyrosinase; TRP, tyrosinase-related protein. *P<0.05.

ments, arbutin (22), a direct inhibitor of tyrosinase activity, at 200 µg/ml completely inhibited α-MSH stimulated tyrosinase activity to basal levels (Fig. 3).

*Effects of soyasaponin Ag on the expression of melanin synthesis factors in α-MSH-stimulated B16F10 cells.* We then examined the effect of soyasaponin Ag treatment on the the expression of tyrosinase, TRP-1 and TRP-2 in B16F10 cells stimulated with α-MSH. As demonstrated by western blot

analysis, soyasaponin did not affect α-MSH-induced tyrosinase and TRP-1 expression (Fig. 4). Of note, treatment of the B16F10 cells with 100 $\mu$M of soyasaponin Ag abolished TRP-2 expression (Fig. 4).

## Discussion

Saponins are beneficial bioactive molecules that are widely distributed in plants and exhibit vast structural and functional diversity; thus, they may be important substances which are worthy of investigation for potential pharmaceutical applications.

The process of melanogenesis is under complex regulatory control by at least 125 genetic loci (23). The enzyme tyrosinase plays a critical role in melanin biosynthesis, and in concert with TRP-1 and TRP-2 is responsible for modifying melanin into different forms (5,24). Thus, the expression level and catalytic activity of these enzymes correlate with melanin production and have been widely investigated with respect to the whitening properties of natural compounds (25). The putative effects of soyasaponin Ag on tyrosinase activity, gene expression, or maturation in melanin biosynthesis during skin pigmentation have not been studied to date, at least to the best of our knowledge. In this study, we demonstrate that soyasaponin Ag inhibits melanogenesis in B16F10 murine melanoma cells via the downregulation of tyrosinase TRP-2 expression. In our initial experiments, we determined that soyasaponin Ag had no cytotoxic effect on B16F10 melanoma cell viability, even at high concentrations. This first indication of the safe use of soyasaponin Ag as an anti-melanogenic agent needs however, to be verified in normal melanocytes and in *in vivo* models.

There are different types of inhibitory mechanisms for melanin synthesis in melanocytes. Indeed, hypopigmenting agents have been classified according to their mechanism of action into three categories: a) regulation of enzyme, which is subdivided into three categories: i) regulation of transcription and maturation of tyrosinase, ii) inhibition of tyrosinase activity, and iii) post-transcriptional control of tyrosinase; b) inhibition of melanosome transfer; and c) additional mechanisms, such as regulation of the melanocyte environment and antioxidant agents (26). Thus, many anti-melanogenic agents, such as kojic acid, hydroquinone and arbutin, are direct inhibitors of tyrosinase enzyme activity (25). Additionally, some compounds exert their anti-melanogenic activities via the suppression of tyrosinase gene expression, although they do not have any direct inhibitory effect on tyrosinase activity in general (27). This type of mechanism was previously identified for acetylsalicylic acid (ASA) in B16 melanoma cells, where ASA exerted a strong dose-dependent inhibitory effect on tyrosinase expression (28). Moreover, ASA did not exert any effect on mushroom tyrosinase activity (28). Other compounds, such as oleoylethanolamide were found to have a more general effect on melanogenesis, inhibiting the expression levels of MITF, TRP-1 and tyrosinase (29). Indeed, MITF plays a crucial role in melanogenesis as the master regulator of tyrosinase, TRP-1 and TRP-2 expression (30). The hydrolyzed ginseng extract, was found to downregulate MITF and TRP-1 expression B16F10 cells, but only indirectly to attenuate cellular tyrosine activity (31). Moreover, the

coumarin derivative, osthol, was found to decrease tyrosinase, TRP-1 and TRP-2 expression, but not to modulate tyrosinase activity (32).

The results of the present study indicate that soyasapogenol A treatment decreased the expression of TRP-2 protein and attenuated melanin production. The most likely explanation for these findings is that soyasapogenol A affects MITF activity/expression, which in other studies has been associated with hypopigmentation (33-38). Importantly, a number of studies have reported that not all skin-whitening agents, such as nicotinic acid hydroxamate, glutathione-monoisopropyl ester and plumbagin, simultaneously inhibit TRP-1 and TRP-2 expression (39-41). In addition, the melanin content can be affected by mutation and degradation of the tyrosinase protein (42).

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that soyasapogenol A inhibits melanogenesis by downregulating the protein levels of TRP-1 and TRP-2, resulting in reduced melanin production. Therefore, given the biological properties of soyasapogenol A, we suggest that saponin Ag is a putative skin-whitening agent and may be effective in the treatment of hyperpigmentation disorders, and may also be used in skin-whitening cosmetics. Further investigations are required in order to fully determine the depigmenting mechanisms of action of soyasapogenol A, as well as its anti-melanogenic activity *in vivo*.

## Acknowledgements

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