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Research Paper

Inhibitory effects of 5-chloroacetyl-2-piperidino-1,3-selenazole, a novel selenium-containing compound, on skin melanin biosynthesis

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Abstract

Objectives Increased production and accumulation of melanin leads to many hyperpigmentation disorders such as melasma, freckles and geriatric pigment spots. Thus, there is a need for the development of depigmenting agents. Based on our previous reports, selenium derivatives as anti-melanogenic lead compounds could be very important. The aim of this study was to investigate the depigmenting effect of novel selenium-containing compounds.
Methods The inhibitory effects of 5-chloroacetyl-2-piperidino-1,3-selenazole (CS1), a novel selenium-containing compound, on melanogenesis were investigated in B16F10 melanoma cells and cultured brownish guinea pig skin tissue with α-melanocyte-stimulating hormone stimulation.
Key findings We found that CS1 inhibited melanin production in B16F10 cells by suppressing tyrosinase activity and its protein expression. In addition, Western blotting analysis revealed that CS1 suppressed the expression of tyrosinase-related protein (TRP)-1 and TRP-2. Therefore, the depigmenting effect of CS1 might have been due to inhibition of tyrosinase activity and expression of melanogenic enzymes. Furthermore, CS1 had inhibitory effects on melanin biosynthesis of primary cultured skin of brownish guinea pig.
Conclusions The results suggested that CS1 could be a useful candidate for the treatment of skin hyperpigmentation.
Keywords 5-chloroacetyl-2-piperidino-1,3-selenazole; α-melanocyte-stimulating hormone; melanogenesis; tyrosinase; tyrosinase-related protein

Introduction

The major role of melanin, which determines mammalian skin and hair colour, is to protect the skin and underlying tissues from UV-induced skin injury. However, the excessive formation and accumulation of melanin in the skin can produce negative effects such as melasma, freckles and geriatric pigment spots.[1,2] Epidermal and dermal hyperpigmentation may be caused by either increased numbers of melanocytes or the altered activity of melanogenic enzymes.[3]

Melanin is secreted by highly specialized cells known as melanocytes, which are located in the basal layer of the epidermis. Melanocytes are dendritic cells in close contact with neighbouring keratinocytes, and together they form the epidermal melanin unit, the function of which is to produce and distribute melanin in the skin.[4–6] The maturation state of a melanosome is ranked on a scale from one to four (I–IV) according to its structure and the capacity, quality and arrangement of melanin formation.[7,8]

In mammals, melanin biosynthesis is affected by a range of stimuli, including UV-B radiation and cyclic AMP (cAMP)-elevating agents.[9–12] Of these stimuli, the binding of α-melanocyte-stimulating hormone (α-MSH) to melanocortin 1 receptor (MC1R) increases the concentration of cAMP, thereby enhancing melanogenesis. cAMP promotes the increased expression of microphthalmia-associated transcription factor (MITF), which contributes to the development and differentiation of melanocytes.[13–15] Melanin

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biosynthesis is mainly regulated by tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2. Tyrosinase plays a significant role in the modulation of melanin production, first by catalysing the hydroxylation of tyrosine to DOPA and second by catalysing the conversion of DOPA to DOPA-quinone. TRP-2 catalyses the rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and of TRP-1-oxidized DHICA to indole-5-6-quinone carboxylic acid.

Two types of melanin are formed in mammals: black/brown eumelanins and yellow/red pheomelanins. Tyrosinase is required for the biosynthesis of eumelanin and pheomelanin. In comparison, TRP-1 and TRP-2 are more critical for the biosynthesis of eumelanin than pheomelanin.[16–18]

Selenium is an essential element in biological activity, and has been reported to be an antioxidant.[19,20] We reported previously that selenium derivatives have anti-melanogenic effects.[21,22] Therefore, we supposed that the novel selenium-containing compounds might be of use as skin-whitening agents. Thus, we tested several novel selenium-containing compounds as candidates for depigmenting agents. Among the 26 different 2-amino-1,3-selenazole and bis-(2-amino-5-selenazoyl) ketone derivatives tested, 5-chloroacetyl-2-piperidino-1,3-selenazole (CS1) suppressed melanin formation the most strongly. Also, we have investigated the depigmenting effect of CS1 on B16F10 melanoma cells and the inhibitory effects of CS1 on melanin biosynthesis in α-MSH-stimulated brownish guinea pig skin.

Materials and Methods

Reagents

The selenium-containing compounds were donated by Dr K. Mamoru of the University of Gifu, Japan. Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin (100 IU/50 μg/ml) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum and bovine calf serum were purchased from JBI (Daegu, Republic of Korea). All other chemicals, including α-MSH, mushroom tyrosinase, arbutin, dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), and L-DOPA were purchased from Sigma (St Louis, MO, USA).

Cell culture and treatment

B16F10 cells, obtained from the American Type Culture Collection (ATCC), were cultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin in air containing 5% CO_2 at 37°C, and the medium was changed every two days. A stock solution of each sample (10 mM in DMSO) was added to the culture at a final concentration of 2.5, 5, 7.5 or 10 μM. After 72 h, the cells were harvested and subjected to various assays.

Assay of melanin content

The melanin content was determined following the methods of Hosoi *et al.*[23] with some modification. B16F10 cells (1×10^5 cells/ml) were cultured with 10 nM α-MSH in six-well plates. After 48 h, the cells were treated with various concentrations of CS1 (2.5–10 μM) for 72 h. After washing with phosphate-buffered saline (PBS), the cells were harvested by trypsinization. The cell pellets were solubilized in 200 μl 1 M NaOH containing 10% DMSO at 80°C for 1 h. The absorbance of each well was then measured at 405 nm using a spectrophotometer. Arbutin, a tyrosinase inhibitor, was used as a positive control.

Determination of the effect of CS1 on cell viability

A cellular proliferation assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). B16F10 cells (4×10^3 cells/ml) were cultured with 10 nM α-MSH in 96-well plates. After 24 h, the cells were treated with various concentrations of CS1 (2.5–10 μM) for 72 h. At the end of the incubation period, 50 μl MTT solution (1 mg/ml in PBS) was added to each well. After incubation at 37°C for 2 h, the medium was gently removed, and 150 μl 0.04 M HCl in isopropanol was added. The absorbance of each well was measured at 570 nm using a spectrophotometer.

Assay of mushroom tyrosinase activity

Each concentration (2.5–10 μM) of test material was prepared in PBS. To each well of a 96-well microplate were added 120 μl L-DOPA (8.3 mM, dissolved in 80 mM phosphate buffer, pH 6.8) and 40 μl of either the same buffer or the test sample, followed by 40 μl mushroom tyrosinase (125 U, dissolved in 80 mM phosphate buffer, pH 6.8). After 30 min at 37°C, the amount of dopachrome in the reaction mixture was measured. The inhibitory activity of the sample was determined based on the optical density at 490 nm.

Assay of B16F10 cell tyrosinase activity

Tyrosinase activity was determined based on the rate of oxidation of L-DOPA. B16F10 cells were lysed in 200 μl PBS containing 1% Triton X-100 and 0.1 mM PMSF and then sonicated on ice for 2 min. After centrifugation at 12 500 rev/min for 15 min, the amount of enzymatic activity in the supernatant was assayed using 150 μg protein for each reaction.

Western blotting

Changes in the expression of various melanogenic proteins, including tyrosinase, TRP-1 and TRP-2, in B16F10 cells treated with 10 μM CS1 were evaluated by Western blotting. After washing with PBS, cells were lysed with ice-cold RIPA buffer containing protease inhibitors and centrifuged at 13 000 rev/min for 20 min at 4°C. The resulting supernatant was collected and assayed for protein concentration using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Samples of the lysates containing 20 μl protein were separated by SDS-PAGE using 10% resolving and 3% acrylamide stacking gels and then transferred to nitrocellulose sheets. Blocking was performed in Tris-buffered saline containing 5% skim milk powder and 0.1% Tween-20. The primary antibodies used were mouse anti-α-tubulin (Sigma), goat anti-tyrosinase, goat anti-TRP-1, and goat anti-TRP-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The intensity of the resultant bands was measured using ImageMaster 2-D Elite software, version 3.10.

Organ culture of guinea pig skin

All experimental procedures were performed according to the Principles of Laboratory Animal Care (NIH publication, #85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. Female brownish guinea pigs (350–600 g each) were kindly donated by the AmorePacific R&D Center (Republic of Korea). Standard organ culture methods were used with a minor modification: whole skin was maintained for five days so that the structure of the epidermis and dermis would closely resemble that of intact skin.[24] Hair was removed from the dorsal skin of the guinea pigs using an electric shaver, and a 4-mm punch biopsy was taken. The subcutaneous fat and lower dermis were carefully removed under a stereomicroscope using a surgical scalpel. The skin samples were placed epithelium-side up on an air–liquid interface using culture inserts (polytetrafluorethylene membranes, filter pore size of 0.4 μm; Millipore Corp., Bedford, MA, USA). The inserts were placed on six-well plates for five days at 32°C in a humidified incubator with 5% CO_2. Organ culture was performed in DMEM containing 10% fetal calf serum and penicillin/streptomycin (100 IU/50 μg/ml), and the medium was changed daily.

Treatment with CS1

The skin biopsies were stabilized and treated with α-MSH, a melanogenic stimulator, for 24 h and then with 1 mM CS1 for 72 h. After three days of survival *ex vivo*, the skin fragments were removed from the inserts, and the effects of CS1 were examined histologically.

Histological analysis

The skin biopsy specimens were fixed in 4% paraformaldehyde at 4°C for 24 h and then dehydrated and embedded in paraffin according to standard procedures. Serial sections (5-μm thickness) were obtained. The melanocytes and melanin were visualized using Fontana-Masson staining. Digital images were collected using an inverted light microscope (Axiovert S 100, Zeiss, Oberko, Germany) attached to a digital CCD camera (Axiocam, Zeiss).

Statistical analysis

The data were analysed using Statistical Analysis System software (PRISM). All the data were expressed as mean ± SD of three independent experiments performed in triplicate. Statistical comparisons between the different treatments were performed using one-way analysis of variance with Tukey's multiple comparison post test. P values of < 0.05 were considered to be statistically significant.

Results

Twenty-six selenium-containing compounds diluted to 10 μM were screened for inhibitory effects on melanin formation. Among the tested compounds, CS1 (compound **17**; Figure 1a)

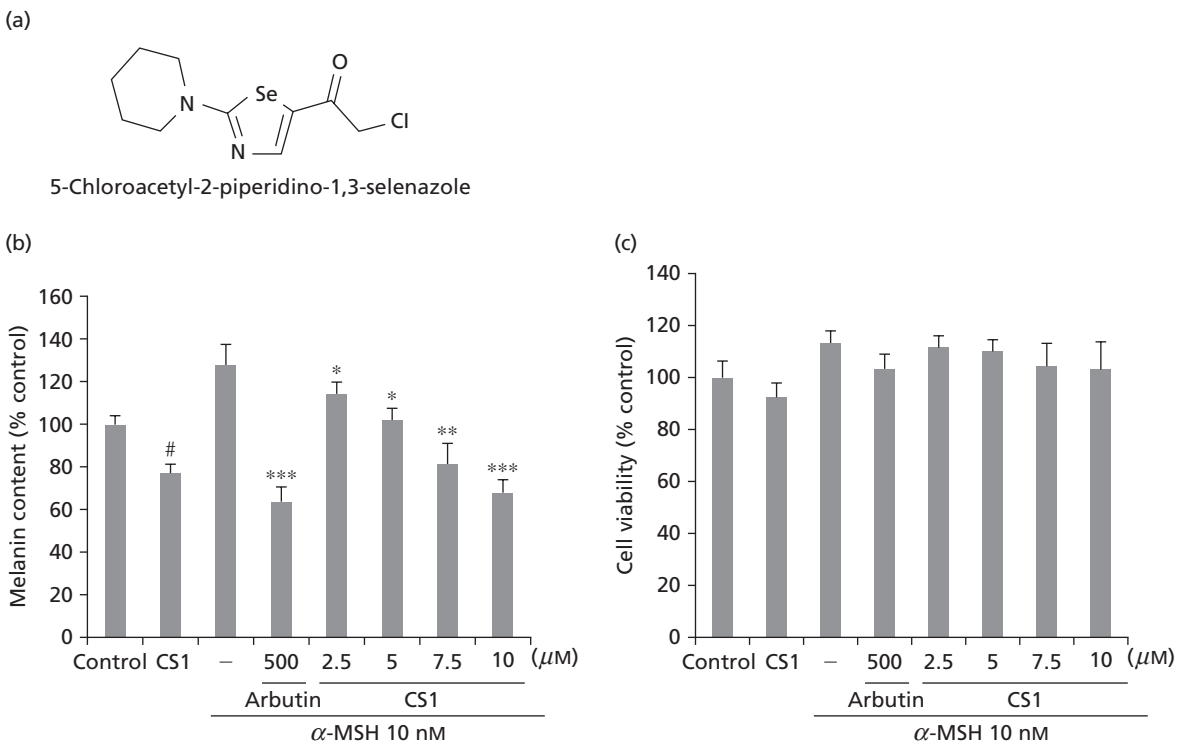

Figure 1 Effects of 5-chloroacetyl-2-piperidino-1,3-selenazole on the melanin content and viability of B16F10 cells. (a) Chemical structure of 5-chloroacetyl-2-piperidino-1,3-selenazole (CS1). (b) The melanin content of B16F10 cells was determined after incubation with various concentrations of CS1 (2.5–10 μM) for 72 h. (c) Cells were treated with various doses of CS1 (2.5–10 μM) and then examined for viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Arbutin was used as a positive control. The data is represented as a mean ± SD of five independent experiments. $^{\#}P < 0.05$ compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with α-melanocyte-stimulating hormone (α-MSH)

Table 1 Effects of selenium-containing compounds on melanogenesis of B16F10 cells

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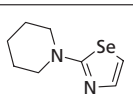
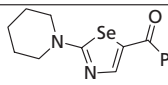
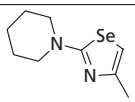
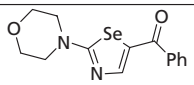
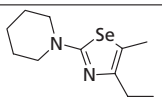
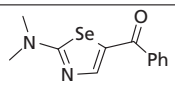
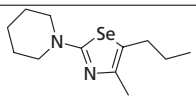
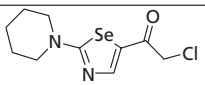
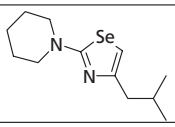
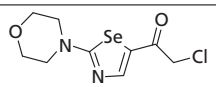
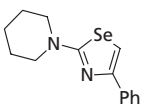
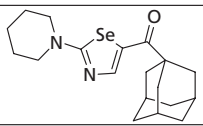
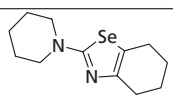
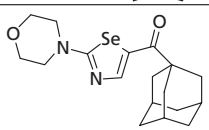
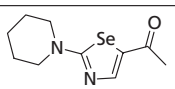
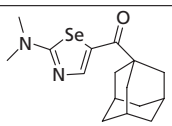
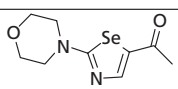
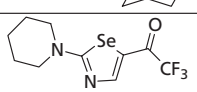
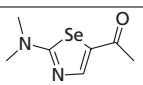
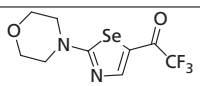
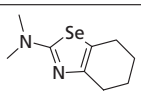
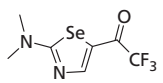
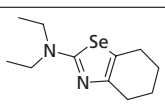
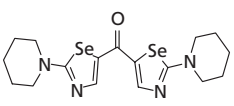
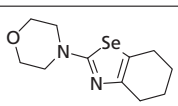
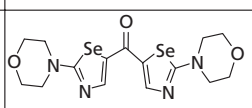
1		na[b]	–	**14**		na	–
2		na	–	**15**		na	–
3		na	–	**16**		na	–
4		na	–	**17**		53.4	10.6
5		na	–	**18**		25	25.5
6		7.4	>50	**19**		na	–
7		na	–	**20**		18	40.7
8		na	–	**21**		8.2	>50
9		na	–	**22**		na	–
10		na	–	**23**		na	–
11		na	–	**24**		7.6	>50
12		na	–	**25**		30.1	19.8
13		na	–	**26**		na	–

[a]Values are mean of inhibition (Inh) of melanin production relative to the α-melanocyte-stimulating hormone control: compounds were used at 10 μM ($n = 4$). [b]na, not active.

showed the highest inhibitory effect on melanin production in B16F10 cells (Table 1).

CS1 at concentrations of 2.5–10 μM reduced melanin production in B16F10 cells in a dose-dependent manner (Figure 1b). In cells exposed to α-MSH alone, the melanin content increased by approximately 30%. CS1 had dose-dependent inhibitory effects on α-MSH-stimulated melanin production. Also, CS1 reduced melanin content in α-MSH unstimulated B16F10 cells. Arbutin, the positive control, inhibited α-MSH-induced melanogenesis (64% at 500 μM). The MTT assay indicated that CS1 was not significantly cytotoxic to B16F10 cells at concentrations of 2.5–10 μM (Figure 1c).

CS1 inhibited the DOPA oxidase activity of mushroom tyrosinase in a dose-dependent manner (Figure 2a). At 10 μM,

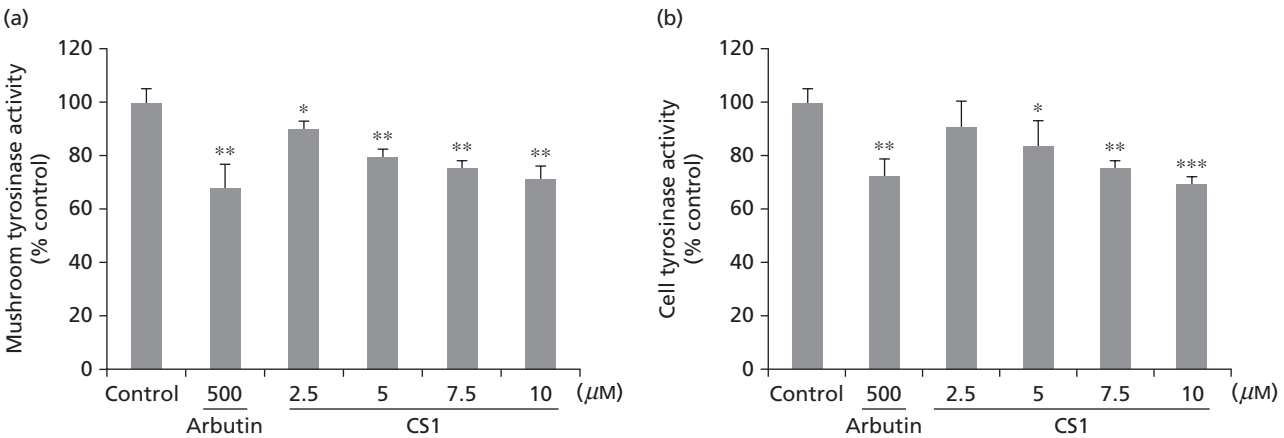


Figure 2 Inhibitory effect of 5-chloroacetyl-2-piperidino-1,3-selenazole on mushroom tyrosinase activity and the tyrosinase activity of B16F10 cells. (a) Tyrosinase activity was determined using L-DOPA as the substrate in a cell-free system. (b) The DOPA oxidase activity of tyrosinase from B16F10 cells was measured using L-DOPA as the substrate. Arbutin was used as a positive control. 5-Chloroacetyl-2-piperidino-1,3-selenazole, CS1. The data are represented as mean ± SD of five independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the control

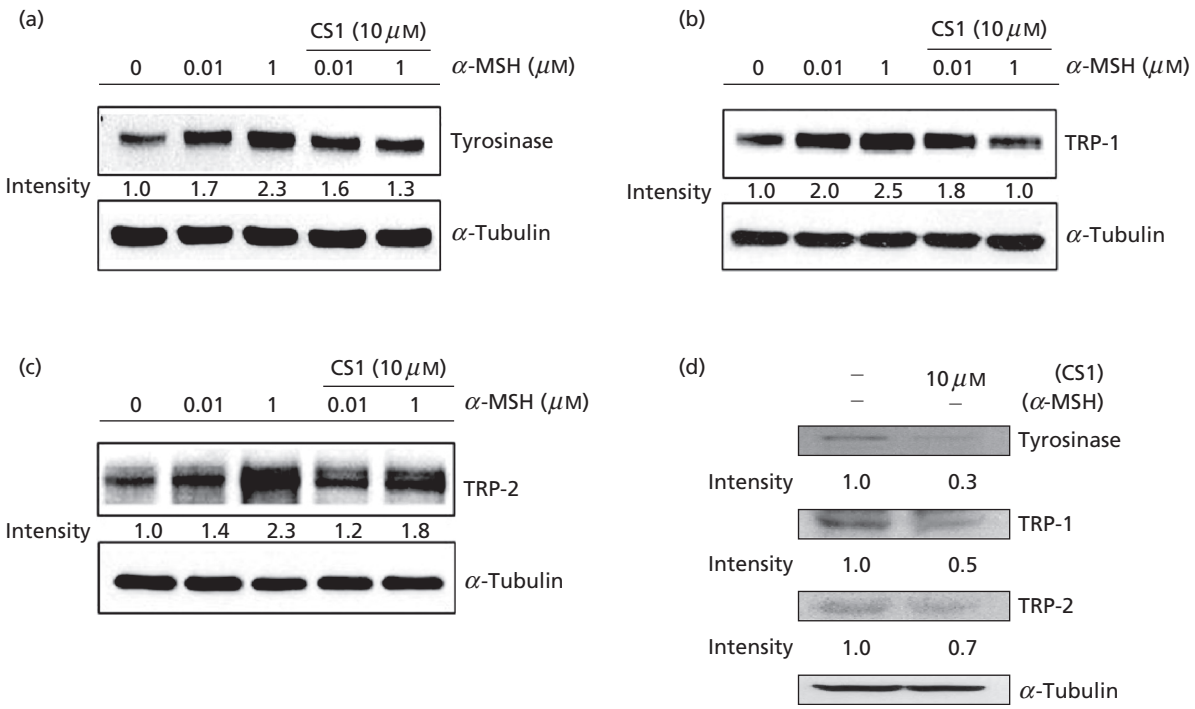


Figure 3 Effect of 5-chloroacetyl-2-piperidino-1,3-selenazole on melanogenic enzyme expression in B16F10 cells. Cells were co-cultured with α-melanocyte-stimulating hormone (α-MSH; 0.01 or 1 μM) and 5-chloroacetyl-2-piperidino-1,3-selenazole (CS1; 10 μM) for 72 h. Whole-cell lysates were subjected to Western blotting using antibodies against (a) tyrosinase, (b) TRP-1, and (c) TRP-2. (d) CS1 suppressed expression of tyrosinase, TRP-1 and TRP-2 in α-MSH-unstimulated B16F10 cells. Equal protein loading was confirmed using anti-α-tubulin antibody. Densitometry analysis of bands was performed as described in the Methods section

CS1 reduced mushroom tyrosinase activity by 28.6%. CS1 also had an inhibitory effect on animal cell-originated tyrosinase activity of B16F10 cells. Tyrosinase activity decreased by 30.2% in B16F10 cells exposed to 10 μM CS1 for 72 h (Figure 2b). Inhibitory effects of the positive control arbutin on tyrosinase activity were observed also.

The treatment of melanocytes with α-MSH increased the expression of melanogenic enzymes. Western blots confirmed that α-MSH (0.01 and 1 μM) stimulated tyrosinase, TRP-1 and TRP-2 expression in B16F10 cells. When co-administered with α-MSH (0.01 or 1 μM), CS1 (10 μM) reduced the upregulation of melanogenic enzyme expression by α-MSH (Figure 3a–c). Also, CS1 suppressed expression of tyrosinase, TRP-1 and TRP-2 in α-MSH-unstimulated B16F10 cells (Figure 3d).

Cultured skin tissue, particularly guinea pig skin, is a more physiologically relevant model of human skin than artificial skin equivalents that contain cultured melanocytes and keratinocytes for testing the hyperpigmentation effect of bioactive compounds.[25,26] In this study, we evaluated the anti-melanogenic effects of CS1 in cultured brownish guinea pig skin tissue. The distribution of melanin in the skin is a critical determinant of skin colour and an indicator of photoprotective efficacy.[27] The transfer and distribution of melanin is modulated by α-MSH.[14] Fontana-Masson staining of skin treated with various concentrations of α-MSH for 24 h revealed an increase of the melanin content compared with control skin. In comparison, treatment with CS1 decreased the melanin content of the epidermis (Figure 4).

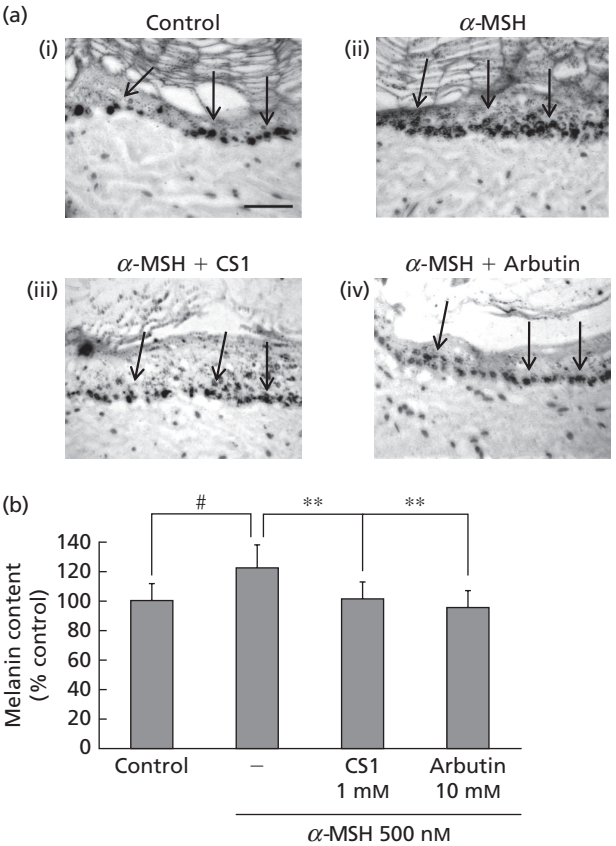


Figure 4 Melanin content visualized by Fontana-Masson staining. (a) Skin was treated for 24 h with α-melanocyte-stimulating hormone (α-MSH), additional treatment with either 5-chloroacetyl-2-piperidino-1,3-selenazole (CS1) or arbutin (positive control) was for a further 72 h. (i) Control; (ii) 500 nM α-MSH; (iii) 500 nM α-MSH and 1 mM CS1; (iv) 500 nM α-MSH and 10 mM arbutin. Black arrows indicate melanin pigment. Scale bar = 20 μm. (b) Quantization of these images by the public domain NIH Image program, Image J. The data are represented as mean ± SD of three independent experiments. $^{\#}P < 0.05$ compared with control, $^{**}P < 0.01$ compared with α-MSH

Discussion

Melanin is synthesized by specialized cells known as melanocytes, which are positioned in the basal layer of the epidermis.[28] Melanin has a number of significant functions, ranging from its role in the determination of phenotypic appearance to protective colouration, balance and auditory processing, the absorption of toxic drugs and chemicals, and neurological development during embryogenesis.[29] Melanogenesis is regulated by UV radiation, which can directly increase tyrosinase activity and expression in melanocytes. UV-induced skin darkening involves an increase in the number of melanocytes, as well as an increase in melanin biosynthesis and melanocyte dendricity, and induces the accumulation of melanosomes in melanocytes and their subsequent transfer to neighbouring keratinocytes.[16,30] However, excessive melanin accumulation can cause various forms of hyperpigmentation, including melasma and age or liver spots, as well as post-inflammatory melanoderma and actinic damage.[31]

The stimulation of melanin synthesis after UV irradiation involves the α-MSH receptor system.[32,33] UV radiation enhances the synthesis and release of α-MSH. α-MSH acts via the MC1R to activate the cAMP pathway. Therefore, α-MSH-stimulated melanogenesis in melanocytes is a suitable model for UV-induced pigmentation. A number of hormones, including adrenocorticotropic hormone (ACTH) and α-MSH, originate from the proteolytic cleavage of propiomelanocortin (POMC). Both ACTH and α-MSH promote proliferation, melanogenesis, and dendrite formation in melanocytes.[34] The α-MSH stimulates eumelanin synthesis, rather than a large increase in melanin production. In response to α-MSH, MC1R stimulates melanogenesis by activating the melanocyte-specific transcription factor MITF. Moreover, MITF upregulates the expression of such melanogenic enzymes as tyrosinase, TRP-1 and TRP-2.[35–37]

Selenium is a required micronutrient in mammals and is found in the form of selenocysteine in several enzymes involved in defence against oxidative stress. We reported previously that selenazole, selenourea and selenoglycoside derivatives could inhibit tyrosinase activity and/or melanin production.[21,22,38] Our results suggested that selenium derivatives may be useful candidates as depigmenting agents.

Melanin synthesis is controlled by at least three enzymes: tyrosinase, TRP1 and TRP2. Among these three enzymes, tyrosinase plays a critical role in melanin biosynthesis. Thus, numerous studies have examined the regulation of tyrosinase in melanocytes. It was reported previously that arbutin, a

well known anti-melanogenic agent, inhibited melanin synthesis by inhibition of tyrosinase activity without the suppression of tyrosinase expression.[39]

In this study, the commercial mushroom tyrosinase was used as an enzyme source because it was readily available. However, tyrosinase obtained from different biological sources may exhibit other structural and functional characteristics. Therefore, it is necessary to assess the activity of melanocyte-originated cellular tyrosinase for the evaluation of skin brightening agents. CS1 directly decreased the activity of mushroom and cell-originated tyrosinase. Also, CS1 regulated the protein expression of multiple melanogenic enzymes.

These results suggested that the depigmenting effect of CS1 was caused by the inhibition of tyrosinase activity and melanogenic enzyme expression, which eventually reduced melanin biosynthesis. In addition, CS1 reduced the distribution of melanin in α-MSH-stimulated brownish guinea pig skin.

Conclusions

CS1 inhibited α-MSH-stimulated melanogenesis in B16F10 cells and cultured brownish guinea pig skin tissue, implying that the depigmenting effect of CS1 may be effective on active melanocytes. The results suggested that compound CS1, a selenium derivative, could be a useful compound as a potential skin-whitening agent.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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