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Research Paper

A derivative of 2-aminothiazole inhibits melanogenesis in B16 mouse melanoma cells via glycogen synthase kinase 3β phosphorylation

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Abstract

Objectives We have investigated whether KHG25855 (2-cyclohexylamino-1,3-thiazole hydrochloride) affected melanogenesis in B16 mouse melanoma cells, and the mechanisms involved.
Methods Melanin content and tyrosinase activity were measured using an ELISA reader after cells were treated with KHG25855. KHG25855-induced signalling pathways were examined using Western blot analysis.
Key findings KHG25855 decreased melanin production in a dose-dependent fashion, but KHG25855 did not directly inhibit tyrosinase, the rate-limiting melanogenic enzyme. The expression of microphthalmia-associated transcription factor, tyrosinase, and the related signal transduction pathways were also investigated. The effects of KHG25855 on the extracellular signal-regulated kinase and cAMP response element binding protein signalling pathways were determined, and KHG25855 was shown to have no effect on these signalling pathways. The Wnt signalling pathway is also deeply involved in melanogenesis, and so glycogen synthase kinase 3β (GSK3β) phosphorylation was assessed after KHG25855 treatment; KHG25855 caused GSK3β phosphorylation (inactivation), but the level of β-catenin was not changed by KHG25855. Furthermore, α-melanocyte stimulating hormone-induced tyrosinase expression was downregulated by KHG25855.
Conclusions We propose that KHG25855 showed hypopigmentary activity through tyrosinase downregulation via GSK3β phosphorylation.
Keywords α-melanocyte stimulating hormone; glycogen synthase kinase 3β; KHG25855; melanogenesis; tyrosinase

Introduction

Human skin shows wide pigment gradations that range from white-to-black. This feature of human skin is due to the amount of a chemically-stable pigment known as melanin, which is produced in the skin.[1] Melanin synthesis takes place within specialized intracellular organelles (melanosomes) that contain tyrosinase, the key enzyme in melanogenesis, and tyrosinase catalyses the rate-limiting reaction of the melanogenic process.[2] Tyrosinase catalyses the oxidation of tyrosine to dopaquinone and the oxidation of 3,4-dihydroxyphenylalanine (DOPA) to dopaquinone.[3] Therefore, melanin production is regulated primarily by the expression and activation of tyrosinase.[4,5]

The production of melanin pigment is stimulated by extracellular signals, such as ultraviolet (UV) light, endothelin 1, α-melanocyte stimulating hormone (α-MSH), and a wide variety of growth factors and cytokines.[1] α-MSH binds to melanocortin 1 receptor, which stimulates melanogenesis through an increase in cAMP, followed by increased expression of microphthalmia-associated transcription factor (MITF).[2] MITF plays an important role as a melanocyte-specific transcription factor in melanocyte development and differentiation.[6] In addition, MITF strongly stimulates tyrosinase expression, which suggests that MITF is an important regulator of melanogenesis.[5,7]

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cAMP response element binding protein (CREB) phosphorylation is known to induce the expression of MITF and to increase melanin synthesis.[8,9] Moreover, several reports indicate that signalling pathways, such as the extracellular signal-regulated kinase (ERK) and Akt pathways, are involved in melanogenesis.[10,11] Thus, inhibition of ERK and Akt results in an increase in melanin synthesis in melanocytes.[12,13]

MITF expression is also regulated by the Wnt signalling pathway. Wnt pathway activation results in glycogen synthase kinase 3β (GSK3β) inactivation and subsequent β-catenin accumulation.[14] Furthermore, the accumulated β-catenin moves into the nucleus and binds lymphoid-enhancing factor/T-cell factor transcription factor, after which MITF expression is stimulated.[15] In contrast, it is known that activated GSK3β leads to the ubiquitination and degradation of β-catenin.[16]

Combinatorial chemistry serves as a powerful tool in lead discovery and optimization by allowing a rapid generation of potential candidates for screening. Lead optimization involves structural modifications of a hit compound to lead compound that has demonstrated the desired biological or pharmacological activity, often using an in-vitro assay system. Among the broad range of templates, heterocyclic scaffolds represent the most promising molecules as leading structures for the discovery of novel synthetic drugs. In particular, a 2-aminothiazole core is found in numerous drugs, and clinical and preclinical candidates that address a broad spectrum of targets.[17] For example, the 2-aminothiazole compounds bind to enzymes, such as cyclooxygenases, kinases, and acetylcholinesterase, and receptors, such as integrins and various members of the G-protein-coupled receptor family.[18–20]

As a part of an effort for exploring new hypopigmentary compounds, we have examined our in-house indigenous chemical library. KHG25855 (2-cyclohexylamino-1,3-thiazole hydrochloride), a 2-aminothiazole derivative, was selected from the primary screening. Thus, we determined the effects of KHG25855 on melanin synthesis and tyrosinase activity using B16 mouse melanoma cells. In addition, we determined the effects of KHG25855 on several signalling pathways related to melanogenesis.

Materials and Methods

Materials

KHG25855 was dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C as a stock solution (50 mM). Synthetic melanin, 3,4-dihydroxy-L-phenylalanine (L-DOPA), (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO), and mushroom tyrosinase were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Antibodies specific for phospho-ERK1/2 (Thr202/Tyr204, #9101S), total (phosphorylated and non-phosphorylated) ERK1/2 (#9102), phospho-CREB (ser133, #9198), total CREB (#9197), phospho-GSK3β (#9336), total GSK3β (#9315), and β-catenin (#9581) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and microphthalmia Ab-1 (C5, MS-771-P0) was obtained from NeoMarkers (Fremont, CA, USA). Secondary antibodies specific for anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000) and anti-rabbit IgG (PI-1000) were purchased from Vector Laboratories (Burlingame, CA, USA).

Synthesis of KHG25855

KHG25855 was synthesized at the Organic Chemistry Laboratory (Korea Institute of Science and Technology, Seoul, Korea). A solution of cyclohexylisothiocyanate (1 g, 7 mmol) in ethanol (15 ml) was added to an ethanolic ammonia solution (28 mmol (7 ml 2.0 mol solution in ethanol)) at room temperature and the reaction mixture was refluxed for 4 h. The reaction mixture was cooled and the precipitation was collected by filtration to afford cyclohexylthiourea (0.78 g (70%)). A solution of cyclohexylthiourea (0.2 g, 1.26 mmol) in acetone (3 ml) was added to 2-chloroacetaldehyde (0.24 ml, 1.26 mmol), and the reaction mixture was refluxed for 24 h. The reaction mixture was cooled and the solvent was removed by evaporation to give a dark blue oily liquid (0.26 g (94%)). This was separated by flash chromatography using silica gel and a mixture of ethyl acetate and n-hexane (2 : 1, v/v) as the eluent to obtain a yellow solid (0.23 g (83%)). Crystallization from ethyl acetate and n-hexane gave a light-yellow solid: mp 217–218°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27~2.31 (m, 10H, C_6H_{11}), 3.14 (methineCH, 1H, C_6H_{11}), 6.46 (d, ^3J = 4.0 Hz, 1H, CH), 7.06 (d, ^3J = 4.0 Hz, 1H, CH), 9.90 (br s, 1H, NH), 13.57 (br s, 1H, HCl).

Cell culture

B16F10 murine melanoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA), 50 μg/ml streptomycin and 50 μg/ml penicillin (Hyclone) in 5% CO_2 at 37°C.

Cell viability assay

Cell viability was determined using a crystal violet assay. After incubating cells with KHG25855 for 24 h, the media were removed, and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times with distilled water, and the crystal violet retained by adherent cells was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

Measurement of melanin content

Extracellular melanin release was measured as described previously, with a slight modification.[21] Briefly, B16F10 cells were incubated at a density of 1×10^5 cells in 6-well plates overnight. α-MSH (1 μM) was then added and cells were treated with increasing concentrations of KHG25855 in phenol red-free DMEM for three days. Samples (200 μl) were then placed in 96-well plates and the optical density (OD) of each culture well was measured using an ELISA reader at 400 nm. The number of cells was then counted using a haemocytometer. Melanin production was expressed as the percentage of α-MSH-treated controls.

Tyrosinase activity

Tyrosinase activity was assayed as DOPA oxidase activity. B16F10 cells were incubated at a density of 1×10^5 cells in

6-well plates and incubated with KHG25855 in DMEM for three days. Cells were washed with phosphate buffered saline (PBS) and lysed with lysis buffer (0.1 M phosphate buffer, pH 6.8, containing 1% Triton X-100). They were then disrupted by freeze-thawing, and lysates were clarified by centrifugation at 13 000 rev/min for 30 min. After quantifying protein content using a protein assay kit (Bio-Rad, Hercules, CA, USA), the cell lysates were adjusted to the same amount of protein with lysis buffer, 90 µl of each lysate was pipetted into the wells of a 96-well plate and 10 µl 10 mM L-DOPA was added. Control wells contained 90 µl lysis buffer and 10 µl 10 mM L-DOPA. After incubation at 37°C for 20 min, dopachrome formation was monitored by measuring absorbance at 475 nm using an ELISA reader.

A cell-free assay system was used to determine the direct effect of KHG25855 on tyrosinase activity. Phosphate buffer (70 µl) containing KHG25855 was mixed with 20 µl 53.7 U/ml mushroom tyrosinase, and 10 µl 10 mM L-DOPA was then added. Following incubation at 37°C for 20 min, the absorbance was measured at 475 nm.

Western blot analysis

Cells were lysed in cell lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Complete; Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA). A 20 µg sample of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes, which were then saturated with 5% dried milk in Tris-buffered saline containing 0.5% Tween 20. Blots were then incubated with the appropriate primary antibodies at a dilution of 1 : 1000, and further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyser (Fuji Film, Tokyo, Japan).

Statistics

The differences between the results ($n = 3$) were assessed using a Kruskal-Wallis test followed by a Dunnett's test. P-values ≤0.01 were considered significant.

Results

Effects of KHG25855 on melanin synthesis and tyrosinase activity

The structure of KHG25855 is shown in Figure 1. B16 cells were treated with KHG25855 at various concentrations (1–100 µM) for 24 h and cell viability was determined using the crystal violet assay. Our results showed that KHG25855 was not cytotoxic to B16 cells at concentrations of 1–100 µM (data not shown). To determine the effects of KHG25855 on melanogenesis, cells were cultured with KHG25855 (1–50 µM) for three days in the presence of α-MSH (1 µM), which increases melanin synthesis, and extracellular melanin release was measured. As shown in Figure 2a, KHG25855 treatment reduced α-MSH-induced melanin synthesis in a dose-dependent manner. Furthermore, KHG25855 treatment significantly reduced tyrosinase activity, which was stimulated by α-MSH (Figure 2b).

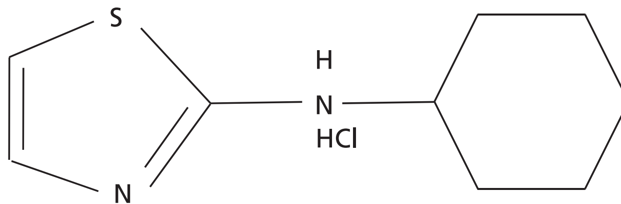

Figure 1 The structure of KHG25855.

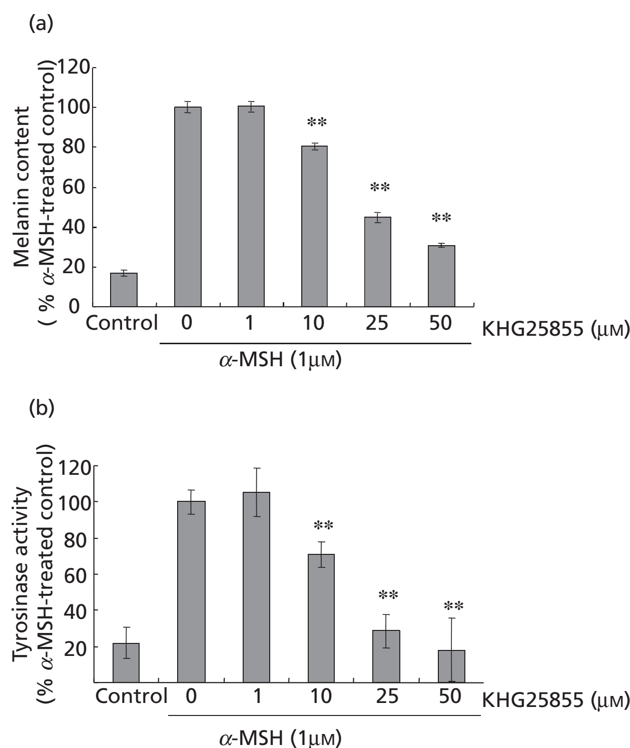

Figure 2 Effects of KHG25855 on melanogenesis in B16 mouse cells. B16 cells were treated with KHG25855 (1–50 µM) in the presence of α-melanocyte stimulating hormone (α-MSH; 1 µM) for three days. Melanin content (a) and tyrosinase activity (b) were measured. The results shown are averages of triplicate experiments ± SD. $^{**}P < 0.01$ compared with α-MSH-treated controls (a and b) and compared with the untreated control.

In addition, we determined whether KHG25855 had a direct effect on tyrosinase activity using mushroom tyrosinase; however, KHG25855 showed no direct effect on tyrosinase activity (data not shown). These results indicated that the inhibitory activity of KHG25855 on melanogenesis may have resulted from inhibition of the melanogenic pathway involving tyrosinase expression, not from the direct inhibition of tyrosinase.

Effects of KHG25855 on the signalling pathways related to melanogenesis

It is known that ERK and CREB phosphorylation regulates melanogenesis. Therefore, we determined whether ERK and

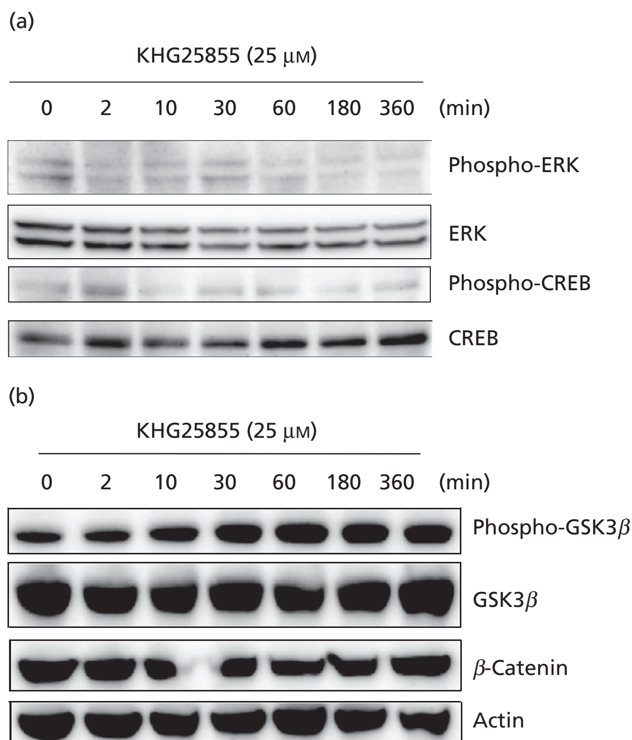

Figure 3 Effects of KHG25855 on the signal transduction pathways in B16 mouse melanoma cells. After 24 h of serum starvation, B16 cells were treated with 25 μM KHG25855 for the times indicated. Whole cell lysates were then subjected to Western blot analysis using antibodies against phospho-specific extracellular signal-regulated kinase (ERK) and phospho-specific cAMP response element binding protein (CREB) (a) or using antibodies against phospho-specific glycogen synthase kinase 3β (GSK3β), total GSK3β, and β–catenin (b). Equal protein loadings were confirmed using ERK, CREB, or actin antibody.

CREB could be affected by KHG25855 in a time-course experiment. KHG25855 showed no effects on ERK and CREB phosphorylation (Figure 3a). In contrast, we showed that GSK3β phosphorylation was clearly induced by 25 μM KHG25855 (Figure 3b). However, we showed that the β-catenin level was not changed by 25 μM KHG25855 (Figure 3b).

Next, we evaluated changes in MITF and tyrosinase protein levels in a time-course experiment after 24–72 h of KHG25855 (25 μM) treatment in the presence of α-MSH (1 μM). Interestingly, MITF levels did not change between 24 and 72 h of KHG25855 treatment compared with α-MSH-treated cells. A slight decrease in MITF was observed at 48 h. In contrast, a clear decrease in tyrosinase protein was demonstrated after 48–72 h of treatment with KHG25855 (Figure 4).

Effects of KHG25855 on GSK3β phosphorylation and melanogenesis

We determined the involvement of GSK3β phosphorylation by KHG25855 in melanogenesis of B16 cells using BIO, a specific GSK3β phosphorylation inhibitor. Cells were treated with KHG25855 for three days in the presence or absence of BIO. BIO was shown to restore KHG25855-induced melanin reduction (Figure 5a). Moreover, BIO blocked

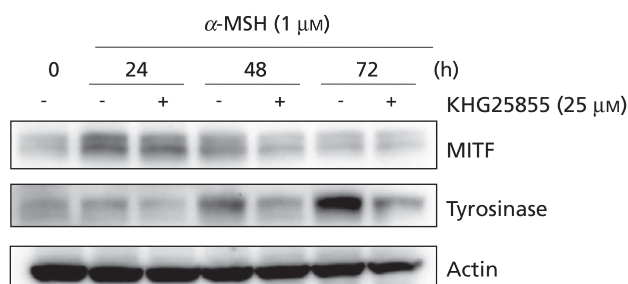

Figure 4 Effects of KHG25855 on the microphthalmia-associated transcription factor and tyrosinase protein expression. B16 mouse melanoma cells were treated with α-melanocyte stimulating hormone (α-MSH; 1 μM) and KHG25855 (25 μM) for 24–72 h. Whole cell lysates were then subjected to Western blot analysis using antibodies against microphthalmia-associated transcription factor (MITF) and tyrosinase. Equal protein loadings were confirmed using anti-actin antibody.

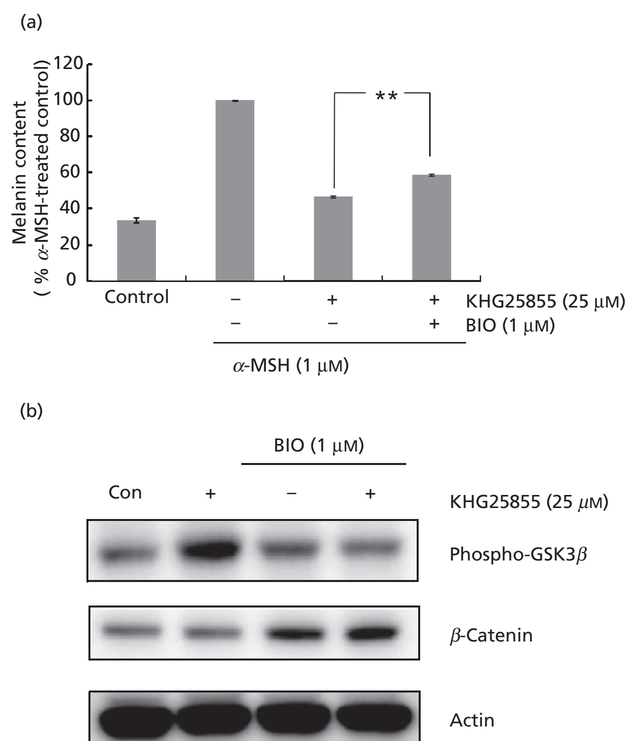

Figure 5 Effects of KHG25855 on the glycogen synthase kinase 3β phosphorylation and melanogenesis. B16 mouse melanoma cells were treated with KHG25855 (25 μM) in the presence or absence of (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO; 1 μM) after adding α-melanocyte stimulating hormone (α-MSH; 1 μM) for three days. (a) Melanin content was measured. Each determination was made in triplicate; the data shown represent the mean ± SD. ** $P < 0.01$ compared with the KHG25855-treated group. (b) Cells were cultured with KHG25855 (25 μM) for 6 h in the presence or absence of BIO (1 μM). Con, control. Whole cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific glycogen synthase kinase 3β (GSK3β) or β–catenin. Equal protein loading was confirmed using actin antibody.

GSK3β phosphorylation in KHG25855-treated cells (Figure 5b). These data clearly indicated that GSK3β phosphorylation was involved in the inhibition of melanogenesis by KHG25855.

Discussion

Exploration of agents that act on the inhibition of melanin synthesis would be of benefit in understanding and studying the precise role of the related signal transduction pathways and their functions in melanogenesis. Thus, the effects of KHG25855, a 2-aminothiazole derivative, on melanin synthesis and the related signalling pathways were studied using B16 mouse melanoma cells.

In this study, we have shown that KHG25855 inhibited melanin synthesis induced by α-MSH in B16 cells. Furthermore, we determined how KHG25855 regulated the molecular mechanism involved in melanogenesis. MITF is a major regulator of the expression of tyrosinase.[22,23] The cAMP pathway is involved in the regulation of melanogenesis, and so we determined whether KHG25855 influenced CREB phosphorylation.[24] Notably, we showed that KHG25855 did not have an effect on CREB phosphorylation (Figure 3a). In addition, several reports have suggested that cAMP upregulation activated ERK in B16 melanoma cells and ERK is an important regulator of melanin synthesis.[25,26] Furthermore, Kim *et al*.[11] reported that ERK activation induced MITF phosphorylation and subsequent degradation; however, KHG25855 did not activate the ERK pathway in this study (Figure 3a). These results indicated that KHG25855 did not decrease melanin synthesis by regulating CREB or ERK activation. We investigated the possibility for direct inhibition of tyrosinase by KHG25855. However, KHG25855 did not inhibit tyrosinase directly. These results indicated that other signalling pathways were involved in KHG25855-induced hypopigmentation.

For the expression of MITF, α-MSH increased the intracellular cAMP level by the activation of adenylate cyclase.[27] Elevated cAMP leads to protein kinase A activation, which results in CREB phosphorylation.[28] This phosphorylated CREB increases MITF expression.[29–31]

MITF expression was also induced by GSK3β inactivation (phosphorylation) and subsequent β-catenin accumulation.[32,33] In contrast, it has been reported that activated (dephosphorylated) GSK3β phosphorylated MITF at Ser298 in melanoma and melanocytes.[34] Furthermore, phosphorylated MITF at Ser298 significantly increased its ability to bind the tyrosinase promoter DNA element, which resulted in increased melanin synthesis.[14,16,34] Thus, we investigated the possible involvement of GSK3β in tyrosinase expression. Our results showed that KHG25855 led to GSK3β phosphorylation (inactivation) without affecting β-catenin expression. In contrast, KHG25855 clearly reduced the tyrosinase protein level, which was increased by α-MSH, whereas KHG25855 had no influence on MITF expression. These results suggested that KHG25855 may have decreased MITF binding affinity to the tyrosinase promoter by GSK3β phosphorylation (inactivation).

Conclusions

We have demonstrated that KHG25855 did not regulate the ERK or CREB pathway, although KHG25855 decreased melanogenesis. Rather, we showed that KHG25855 led to GSK3β phosphorylation, which may have reduced the MITF binding affinity and subsequently decreased the tyrosinase level. We propose that KHG25855 may be useful as a new skin-whitening agent which decreases the level of tyrosinase.

Declarations

Conflict of interest

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

Funding

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