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The hypopigmentary action of KI-063 (a new tyrosinase inhibitor) combined with terrein

Dong-Seok Kim, Sangku Lee, Hyun-Kyung Lee, Seo-Hyoung Park, In-Ja Ryoo, Ick-Dong Yoo, Sun-Bang Kwon, Kwang Jin Baek, Jung-Im Na, Kyoung-Chan Park

Department of Biochemistry, College of Medicine, Chung-Ang University, 221 Heukseok-Dong Dongjak-Gu, Seoul 156-756, Republic of Korea

Dong-Seok Kim, Kwang Jin Baek

National Cosmeceutical Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Oeun-Dong, Yuseong-Gu, Daejeon 305-333, Korea

Sangku Lee, In-Ja Ryoo, Ick-Dong Yoo

Department of Dermatology, Seoul National University College of Medicine, 28 Yongon-Dong, Chongno-Gu, Seoul 110-744, Republic of Korea

Hyun-Kyung Lee, Seo-Hyoung Park, Jung-Im Na, Kyoung-Chan Park

Welskin Co. Ltd, 28 Yongon-Dong, Chongno-Gu, Seoul 110-744, Korea

Sun-Bang Kwon

Correspondence: Kyoung-Chan Park, Department of Dermatology, Seoul National University Bundang Hospital, 300 Gumi-Dong, Bundang-Gu, Seongnam-Si, Kyoungki-Do 463-707, Korea. E-mail: gcpark@snu.ac.kr

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Abstract

Resorcinol derivatives are known to inhibit melanin synthesis. In this study, resorcinol derivatives were synthesized and screened for their activity on melanogenesis. KI-063 (a tyrosinase inhibitor) was examined for its effects on melanogenesis using a spontaneously immortalized mouse melanocyte cell line (Mel-Ab). In a cell-free system, KI-063 directly inhibited tyrosinase, the rate-limiting melanogenic enzyme. Moreover, in a cell system, it inhibited melanin synthesis in a concentration-dependent manner. In addition, KI-063 inhibited the activity of cellular tyrosinase. Thus, this study examined the effects of a combination of KI-063 with terrein, an agent that down-regulates microphthalmia-associated transcription factor. The data suggest that KI-063 has an additive effect in combination with terrein. Thus, the suppression of tyrosinase activity by KI-063 and the inhibition of tyrosinase production by terrein appear to be an optimal combination for skin whitening.

Introduction

The production and distribution of melanin pigment is a major determinant of skin and hair colour. Tyrosinase is a major melanosomal enzyme in melanogenesis, which catalyses the rate-limiting reaction of the melanogenic process (Hearing & Jimenez 1989; Slominski et al 2004). Therefore, melanin production depends mainly on the expression and activation of tyrosinase (Hearing & Tsukamoto 1991). Previous studies reported that resorcinol (1,3-dihydroxybenzene) derivatives inhibit the activity of tyrosinase (Tasaka et al 1998). Compounds with a 4-substituted resorcinol skeleton are potent inhibitors of tyrosinase (Shimizu et al 2000; Kim & Uyama 2005; Song et al 2007). Some of these polyphenols can act as both inhibitors and substrates through a complex reaction involving tyrosinase (Espin et al 1997; Fenoll et al 2003). Indeed, several polyphenols act as suicide substrates, causing a mechanism-based inactivation of tyrosinase (Garcia Canovas et al 1987; Tudela et al 1988; Haghbeen et al 2004; Chang 2007). Moreover, we have recently reported that 4-*n*-butylresorcinol strongly inhibits melanogenesis (Kim et al 2005), and this agent has been useful as an experimental treatment for melasma (Khemis et al 2007). Thus, resorcinol derivatives (including KI-063) were synthesized and screened for their inhibitory effects on tyrosinase.

Microphthalmia-associated transcription factor (MITF) is known to regulate tyrosinase expression and melanin synthesis at the transcription level (Yasumoto et al 1997; Bertolotto et al 1998; Lin & Fisher 2007). Hence, mutations in the MITF gene cause abnormal pigmentation of the skin and hair (Hughes et al 1994; Tassabehji et al 1994; Tachibana 1997). Activation of the extracellular signal-regulated kinase (ERK) pathway has been shown to decrease levels of MITF and to inhibit melanin synthesis (Englaro et al 1998; Kim et al 2002, 2003). We have shown that terrein, a bioactive fungal metabolite, down-regulates MITF and tyrosinase through activation of ERK and thus reduces melanin synthesis (Park et al 2004). We therefore hypothesized that the inhibition of tyrosinase

production by terrein might strongly potentiate the inhibitory effect of tyrosinase inhibitors on melanin synthesis.

This study examined the effects of KI-063 on melanin synthesis and tyrosinase activity in a spontaneously immortalized mouse melanocyte cell line (Mel-Ab), alone and in combination with terrein.

Materials and Methods

Materials

KI-063 and terrein were synthesized at the Korea Research Institute of Bioscience and Biotechnology (Lee et al 2005). Cholera toxin (CT), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), synthetic melanin, L-DOPA, and mushroom tyrosinase were obtained from Sigma (St Louis, MO, USA).

Synthesis of KI-063

KI-063 was synthesized through the esterification of dihydrocaffeic acid with 4-n-butylresorcinol using *N*, *N*′-dicyclohexylcarbodiimide and 4-di(methylamino)pyridine. *N*, *N*′-dicyclohexylcarbodiimide (720 mg, 3.49 mM) and 4-di(methylamino)pyridine (60 mg, 0.49 mM) were added to a solution of dihydrocaffeic acid (500 mg, 2.75 mM) and 4-n-butylresorcinol (450 mg, 2.71 mM) in ethyl acetate (30 mL) at room temperature. The reaction mixture was stirred for 24 h, concentrated, and purified by silica gel column chromatography (2:1 hexane/ethyl acetate) to give KI-063. Its chemical structure was analysed by NMR as follows: ^{1}H NMR ($CDCl_3$, 300 MHz), δ (ppm) 7.04 (d, $J = 8.4$ Hz, 1H), 6.78 (d, $J = 8.4$ Hz, 1H), 6.76 (d, $J = 3.0$ Hz, 1H), 6.68 (dd, $J = 8.1$, 1.8 Hz, 1H), 6.62 (dd, $J = 8.1$, 2.7 Hz, 1H), 6.41 (d, $J = 2.4$ Hz, 1H), 5.19 (br s, 3H), 2.96 (t, $J = 6.6$ Hz, 2H), 2.84 (t, $J = 6.6$ Hz, 2H), 2.33 (t, $J = 7.8$ Hz, 2H), 1.45 (m, 2H), 1.26 (m, 2H), 0.88 (t, $J = 7.5$ Hz, 3H).

Cell cultures

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin (Dooley et al 1994). Mel-Ab cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 nM TPA, 1 nM CT, 50 μg mL^{-1} streptomycin, and 50 U mL^{-1} penicillin at 37°C in 5% CO_2.

Cell viability

Cell viability was determined using a crystal violet assay (Dooley et al 1994). After incubating with KI-063 for 24 h, the culture medium was removed and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature, then rinsed four times with distilled water. The crystal violet retained by the adherent cells was then extracted with 95% ethanol. The absorbance was determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria).

Measurement of melanin content and microscopy

The melanin content was measured using a slight modification of the method described by Tsuboi et al (1998). Briefly, the cells were treated with KI-063 at various concentrations for 4 days. The cell pellets were dissolved in 1 mL 1 M NaOH at 100°C for 30 min and centrifuged for 20 min at 16,000 *g*. The optical density of the supernatants was measured at 400 nm using an ELISA reader (TECAN). Before measuring the melanin content, the cells were observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed with a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Inc., Tucson, AZ, USA) supported by RS Image software (Roper Scientific).

Tyrosinase activity

The tyrosinase activity was assessed using a slight modification of the method described by Busca et al (1996). Briefly, Mel-Ab cells were cultured in six-well plates and incubated with KI-063 at various concentrations for 4 days. The cells were then washed with ice-cold phosphate-buffered saline and lysed with 0.1 M phosphate buffer (pH 6.8) containing 1% Triton X-100. Cells were then disrupted by freezing and thawing, and the lysates clarified by centrifugation at 10 000 *g* for 5 min. After quantifying protein levels and adjusting the concentrations with lysis buffer, 90 μL of each lysate containing the same amount of protein was placed in each well of a 96-well plate and 10 μL 10 mM L-DOPA added to each well. The control wells contained 90 μL 0.1 M phosphate buffer (pH 6.8) and 10 μL 10 mM L-DOPA. After incubation at 37°C, the absorbance at 475 nm was measured every 10 min for at least 1 h using an ELISA plate reader.

A cell-free assay system was used to examine the direct effects of KI-063 on tyrosinase activity. Phosphate buffer (70 μL) containing various concentrations of KI-063 was combined with 20 μL 10 μg mL^{-1} mushroom tyrosinase, and 10 μL 10 mM L-DOPA was added to each well. After incubation at 37°C, the absorbance was measured at 475 nm.

Statistical analysis

Differences between groups were assessed using the Kruskal–Wallis test followed by Dunnett's test. *P* values below 0.01 were considered significant.

Results

Cytotoxicity of KI-063 in Mel-Ab cells

KI-063 is a resorcinol derivative prepared from 4-n-butylresorcinol and dihydrocaffeic acid (Figure 1). To determine whether KI-063 can be used safely as a skin-whitening agent, Mel-Ab cells were treated with 0.01–50 μM KI-063, and the cell viability measured using a crystal violet assay. KI-063 had no cytotoxic effect in Mel-Ab cells at the concentrations used (data not shown).

KI-063 decreased melanogenesis in Mel-Ab cells

To investigate the hypopigmentation effect, KI-063 (0.1–50 μM) was added to cultured Mel-Ab cells for 4 days. The Mel-Ab cells were then photographed under a phase-contrast microscope. Cells treated with KI-063 showed much

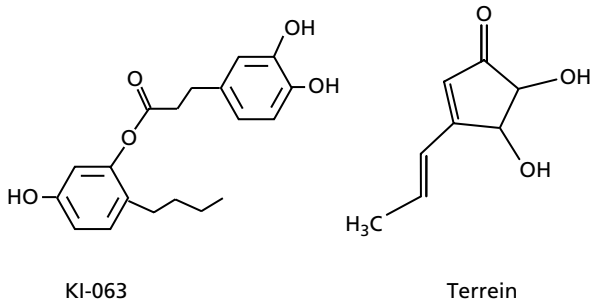

Figure 1 The structures of KI-063 and terrein.

less pigmentation than untreated cells (Figure 2). Melanin content was measured after 4 days' treatment with KI-063. Consistent with the microscopic observations, KI-063 (10–50 μM) significantly inhibited melanogenesis (Table 1). Tyrosinase is a critical enzyme in melanogenesis and is involved in the rate-limiting step. We therefore measured tyrosinase activity after treatment with KI-063 (same concentrations), which was found to decrease as a function of the KI-063 dose (Table 1). This suggests that KI-063 inhibits tyrosinase activity and subsequently suppresses melanin synthesis in Mel-Ab cells.

KI-063 directly inhibited tyrosinase in a cell-free system

To determine whether KI-063 inhibits tyrosinase activity directly, its effect on mushroom tyrosinase was measured in a cell-free system. These results showed that KI-063 had a strong concentration-dependent inhibitory effect on tyrosinase activity (Table 1). This indicates that KI-063 can inhibit melanogenesis, possibly through direct inhibition of tyrosinase.

Combination of KI-063 with terrain

We have previously reported that terrein down-regulates MITF and tyrosinase and thus reduces melanin synthesis (Park et al 2004). KI-063 (10 or 20 μM) was used in combination with terrein (1, 10 or 20 μM) to examine the combined effects of the tyrosinase inhibitor and the tyrosinase production inhibitor. Terrein had no cytotoxic effect on Mel-Ab cells at the concentrations used (Park et al 2004). After 4 days, the Mel-Ab cells were photographed using phase-contrast microscopy. Cells co-treated with KI-063 and terrein showed much less pigmentation than cells treated with KI-063 or terrein alone (Figure 3). Melanin content and tyrosinase activity were measured after 4 days' treatment with KI-063 and terrein. Consistent with the microscopic observations, the combination of KI-063 and terrein had an additive effect and significantly inhibited melanin synthesis and the tyrosinase activity, respectively, at concentrations above 10 μM (Figures 4 and 5). The addition of 1 μM terrein to KI-063 did not have any synergistic effects. This suggests that the potent inhibition of tyrosinase activity by KI-063 works in combination with the reduced level of tyrosinase induced by terrein.

Discussion

Tyrosinase is a key enzyme that regulates the rate-limiting step of melanin synthesis (Ando et al 2007). In addition, resorcinol derivatives have been reported to inhibit tyrosinase activity (Tasaka et al 1998). KI-063 is a synthetic resorcinol derivative. In this study, KI-063 inhibited tyrosinase activity and, as a result, significantly suppressed melanin synthesis. Thus, it appears that a decrease in tyrosinase activity may contribute to the lower pigment content in the KI-063-treated cells.

MITF is known to regulate the expression of tyrosinase (Busca & Ballotti 2000; Tachibana 2000; Lin & Fisher 2007).

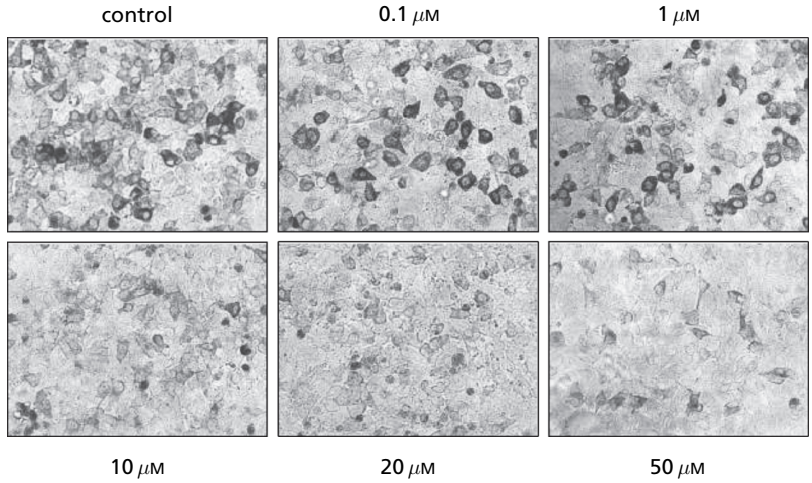

Figure 2 Phase-contrast photomicrographs showing the effects of KI-063 on melanin synthesis in Mel-Ab cells (cultured with 0.1–50 μM KI-063 for 4 days).

Table 1 The effects of KI-063 on melanin synthesis and tyrosinase activity in Mel-Ab cells

	KI-063 concn (μM)						
	0	0.01	0.1	1	10	20	50
Melanin content	100.0 ± 3.6	–	108.5 ± 3.7	98.8 ± 6.7	85.2 ± 2.2*	76.6 ± 3.9*	47.7 ± 2.9*
Tyrosinase activity							
Mel-Ab cells	100.0 ± 6.0	–	94.7 ± 9.7	79.4 ± 12.3	60.8 ± 11.1*	34.4 ± 10.7*	14.5 ± 17.2*
Cell-free system	100.0 ± 5.7	79.4 ± 1.6*	70.2 ± 5.3*	65.6 ± 3.3*	50.2 ± 10.5*	40.5 ± 15.6*	40.9 ± 2.3*

Values are % of control activity, mean ± s.d. of three replicates. *$P < 0.01$ vs untreated control.

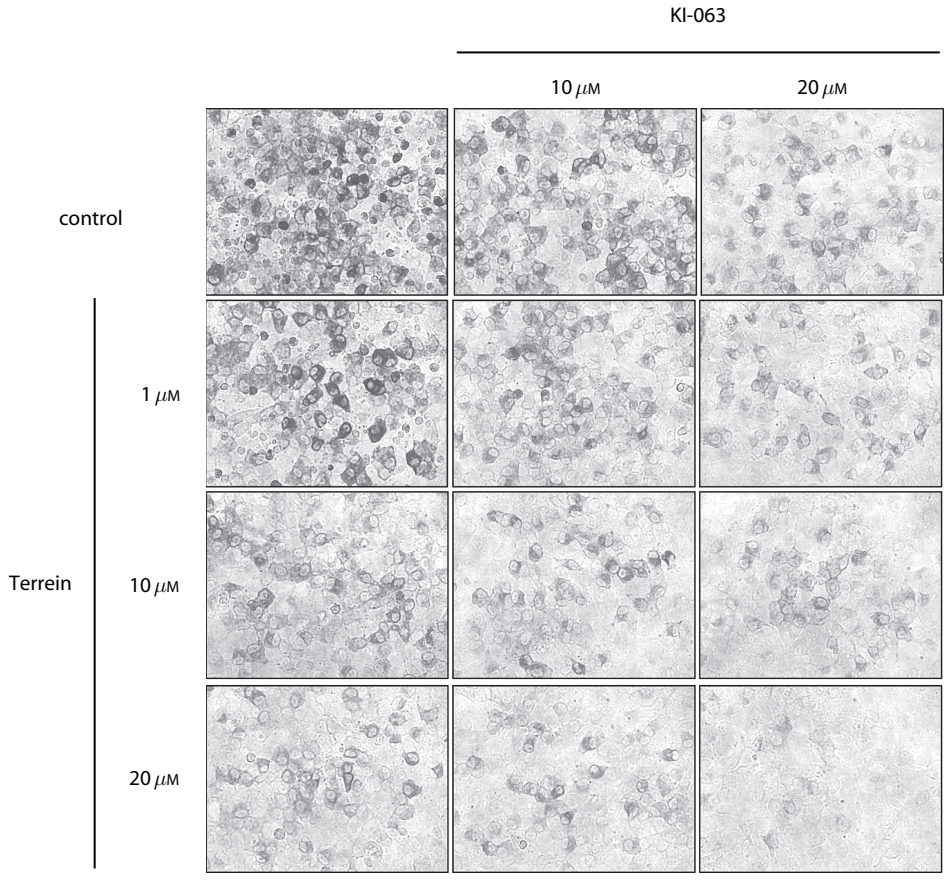

Figure 3 Phase-contrast photomicrographs showing the effects of KI-063 and terrein on melanin synthesis in Mel-Ab cells (cultured with 10 or 20 μM KI-063 without or with 1, 10, or 20 μM terrein for 4 days).

Previous studies suggest that ERK is an important kinase involved in melanin synthesis, as ERK activation induces the phosphorylation and subsequent degradation of MITF (Hemesath et al 1998; Wu et al 2000; Xu et al 2000). In addition, ERK activation by terrein down-regulates MITF, which results in reduced tyrosinase levels and reduced melanogenesis (Park et al 2004). However, terrein does not inhibit the activity of tyrosinase directly. In this study, it was found that KI-063 directly inhibits tyrosinase (Figure 5). Although the mechanism by which KI-063 inhibits tyrosinase is unclear, the structure of KI-063 suggests that it may act as a competitive inhibitor.

Many studies have focused on tyrosinase inhibitors for the development of skin-whitening agents. Many such inhibitors have been developed from studies using model enzymes such as mushroom and plant tyrosinases (Kim & Uyama 2005; Chang 2007). Although these inhibitors have hypopigmenting effects in-vitro, only a few substances were found to have effective hypopigmenting activity in clinical trials. Moreover, they often induce skin irritation or permanent

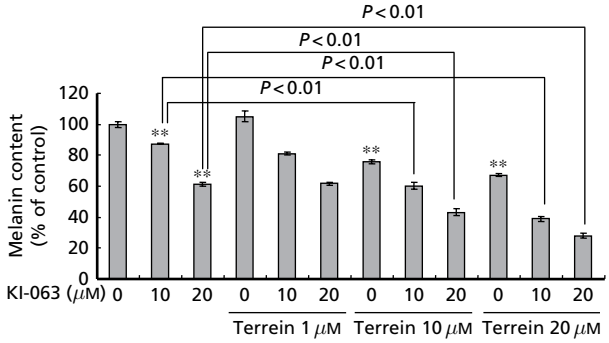

Figure 4 Effects of KI-063 and terrein on melanin content of Mel-Ab cells cultured with 10 or 20 μM KI-063 without or with 1, 10, or 20 μM terrein for 4 days. Data are mean ± s.d. of three replicate experiments. $^{*}P < 0.01$ vs untreated control.

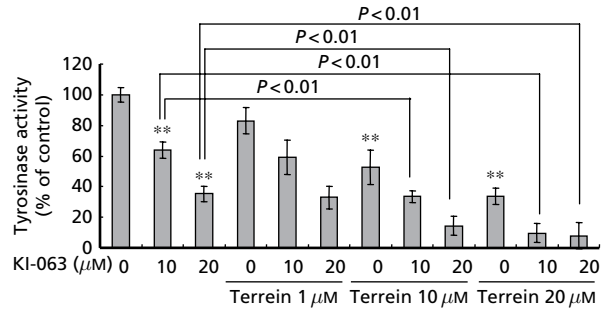

Figure 5 Effects of KI-063 and terrein on tyrosinase activity in Mel-Ab cells cultured with 10 or 20 μM KI-063 without or with 1, 10, or 20 μM terrein for 4 days. Data are mean ± s.d. of three replicate experiments. $^{*}P < 0.01$ vs untreated control.

depigmentation. In addition, some inhibitors require unacceptably high doses to produce visible effects. The skin is a very complex organ, containing various types of cells such as keratinocytes, melanocytes and fibroblasts. Thus, the development of an effective hypopigmenting agent will require further testing of potential candidates for their effects in melanocyte–keratinocyte co-culture models, melanocyte-containing skin-equivalent models, and in-vivo models. Two or more agents that act through different mechanisms are likely to have synergistic hypopigmenting effects (Solano et al 2006). Thus, regulators of signalling pathways have been investigated, and several molecules, such as terrein, have been found to suppress the activity of tyrosinase indirectly.

The current study showed that a combination of KI-063 and terrein dramatically decreased melanin synthesis. This combination allows a reduction in the effective dose of each agent, and is therefore likely to decrease the number of unwanted adverse effects (Solano et al 2006). Hence, a combination of these agents might be a new strategy for the treatment of hyperpigmentory skin diseases.

Conclusions

These results showed that a combination of KI-063 and terrein acted synergistically, indicating that the strong tyrosinase inhibitory activity of KI-063 works in combination with the reduced level of tyrosinase due to terrein. Because melanogenesis in the skin is complex and involves different stages of regulation, this combination suggests a new concept for skin-whitening agents. Future studies should consider a combination of KI-063 and MITF regulators to maximize the hypopigmentary effect.

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