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RESEARCH ARTICLE

Anti-melanogenic effect of (*Z*)-5-(2,4-dihydroxybenzylidene) thiazolidine-2,4-dione, a novel tyrosinase inhibitor

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Abstract We synthesized (*Z*)-5-(2,4-dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498) as a potential tyrosinase inhibitor. MHY498 potently inhibited mushroom tyrosinase activity (mean $IC_{50} = 3.55$ μM) in a dose-dependent manner. MHY498 was more potent than the well-known tyrosinase inhibitor, kojic acid (mean $IC_{50} = 22.79$ μM). When tested in B16F10 melanoma cells treated with α-melanocyte stimulating hormone (α-MSH), MHY498 inhibited murine tyrosinase activity and decreased melanin production without inducing cytotoxicity. Docking models showed that the binding affinity of MHY498 to tyrosinase was higher than that of kojic acid, and docking simulation results indicated that the tyrosinase binding moieties of MHY498 and kojic acid were similar. Western blotting showed that tyrosinase inhibition by MHY498 partly resulted from the expressional modulations of tyrosinase and its transcription factor, microphthalmia-associated transcription factor, via the cAMP–PKA–CREB pathway. These findings suggest that MHY498 could be useful as an anti-melanogenic agent for the prevention and treatment of diseases associated with skin pigmentation.

Keywords (*Z*)-5-(2,4-Dihydroxybenzylidene) thiazolidine-2,4-diones · Melanogenesis · Tyrosinase inhibitor · B16F10 melanoma cells · cAMP · PKA

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Introduction

Skin pigmentation is commonly induced by ultraviolet (UV) light, and is due to an accumulation of melanin in the epidermis. Melanin synthesis takes place within specialized intracellular organelles called melanosomes. In mammals, two major types of melanin are produced, the eumelanins, which are black/brown, and the pheomelanins, which are yellow/red.

Although melanogenesis is a necessary defense mechanism against UV irradiation and excessive oxidative stress, abnormal accumulations of melanin can cause hyperpigmentation, including melasma, freckles, and senile lentigines (Picardo et al. 1999; Brenner and Hearing 2008). In fact, UV radiation, chronic inflammation, and the release of abnormal α-melanocyte stimulating hormone (α-MSH) are well-known triggering factors of hyperpigmentation and inflammatory pigmentation (Choi et al. 2005; Im et al. 2002).

For melanin production, specific enzymes are required, and the best characterized are tyrosinase, tyrosinase-related protein 1 and dopachrome tautomerase. Tyrosinase is a

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copper-containing enzyme and catalyzes the two initial rate-limiting reactions of melanogenesis, that is, the hydroxylation of tyrosine to give 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPAquinone (Busca and Ballotti 2000; Ebanks et al. 2009).

Because of its key role in melanogenesis, tyrosinase is an attractive target for research on the biochemistry of melanogenesis and management of hyperpigmentary disorders. Over the last few decades, various tyrosinase inhibitors, such as, hydroquinone (Garcia and Fulton 1996), ascorbic acid derivatives (Kameyama et al. 1996), azeleic acid (Hermanns et al. 2002), retinoids (Yoshimura et al. 2001), arbutin (Nakajima et al. 1998), kojic acid (Cabanes et al. 1994), hydroxystilbene compounds (e.g. resveratrol) (Ohguchi et al. 2003b; Shin et al. 1998; Kim et al. 2002), and polyphenolic compounds (Ohguchi et al. 2003a; Roh et al. 2004; No et al. 2004) have been reported. However, some well-known tyrosinase inhibitors, including hydroquinone and kojic acid, have limited applications, because of their undesirable side effects, which include dermatitis and cytotoxicity. Therefore, safer and more effective agents are needed.

In this context, our laboratory has reported that compounds with imidazole ring have significant inhibitory activities against tyrosinase (Ha et al. 2011b). In addition, we found that 2-hydroxy, 4-hydroxy or 2,4-dihydroxy phenyl compounds had more potent inhibitory activities against tyrosinase than other derivatives (Chung et al. 2012; Ha et al. 2011a, c). On the basis of these results, we synthesized the compound (*Z*)-5-(2,4-dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498) (Fig. 1), which was substituted NH to S in imidazole ring based on their bioisostere and had 2,4-hydroxy phenyl moiety to expect the potent inhibitory activity against tyrosinase. It has been reported that the synthesis and inhibitory effects of (*Z*)-5-(2,4-dihydroxybenzyli dene)thiazolidine-2,4-dione (MHY498) on Mur ligase and oxidative stress (Cacic and Molnar 2011; Jiang et al. 2011; Ha et al. 2012a; Shah and Singh 2012; Tomasić et al. 2010), however, this is the first report on the anti-melanogenic effect of MHY498.

In this study, we investigated the inhibitory effects of MHY498 on tyrosinase activity and melanin production, and its regulatory effects on melanogenesis in B16F10 melanoma cells. Molecular studies of the inhibitory mechanism of MHY498 on tyrosinase active site were conducted using kinetic study and docking simulation.

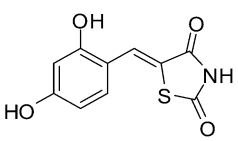

Fig. 1 Chemical structure of (*Z*)-5-(2,4-dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498)

Materials and methods

Synthesis of (*Z*)-5-(2,4-dihydroxybenzylidene) thiazolidine-2,4-dione (MHY498)

To a stirred solution of substituted 2,4-dihydroxybenzaldehyde (200 mg, 1.45 mmol) and thiazolidine-2,4-dione (204 mg, 1.74 mmol) in ethanol (4 mL) was added piperidine (0.03 mL, 0.3 mmol).The reaction mixture was then refluxed for 24 h (Fig. 2). After cooling, water was added, and the precipitate so formed was filtered through a Buchner funnel and washed with water/ethanol (5:1). For further purification, the precipitate was subjected to flash silica gel column chromatography using chloroform/methanol (10:1) as eluent to give (*Z*)-5-(2,4-dihydroxybenzylidene) thiazolidine-2,4-dione (MHY498, 156.1 mg, 45.4 %) as a dark yellow solid.

Melting point, 169.4–171.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.36 (br s, 1 H, NH), 10.40 (s, 1 H, OH), 10.13 (br s, 1 H, OH), 7.93 (s, 1 H, vinylic H), 7.13 (d, 1 H, $J = 8.8$ Hz, 6′-H), 6.38 (d, 1 H, $J = 2.4$ Hz, 3′-H), 6.36 (dd, 1 H, $J = 8.8$, 2.4 Hz, 5′-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.1 (C2), 168.5 (C4), 162.3 (C4′), 160.0 (C2′), 130.6 (benzylic C), 128.0 (C6′), 117.6 (C5), 112.3 (C1′), 109.0 (C5′), 103.2 (C3′); LRMS(ES) *m*/*z* 236 $(M–H)^-$.

Materials

Antibodies against tyrosinase, microphthalmia-associated transcription factor (MITF), phosphorylated Ser96 PKA regulatory type IIα subunit (pPKA RIIα(Ser96)), β-actin, and transcription factor IIB (TFIIB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mushroom tyrosinase, L-tyrosine, 3,4-dihydroxyphenylalanine (L-DOPA), kojic acid, α-melanocyte stimulating hormone (α-MSH), and other chemical reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

Cell culture system

B16F10 cells (from the Korean Cell Line Bank) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Tokyo) containing 10 % fetal bovine serum (FBS, Sigma Chemical Co., USA) and penicillin/streptomycin (100 IU/50 μg/mL) in a humidified atmosphere containing 5 % CO_2 (in air) at 37 °C. B16F10

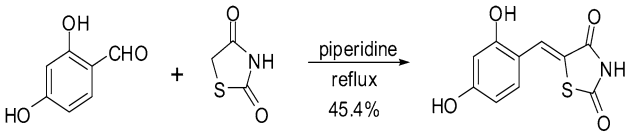

Fig. 2 Brief scheme of the synthesis of MHY498

cells were cultured in 24-well plates for melanin quantification and enzyme activity assays.

Cell viability

Cell survival was quantified by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay that measured mitochondrial activity in viable cells. This method is based on the conversion of MTT (Sigma Chemical Co., USA) to MTT-formazan crystals by a mitochondrial enzyme, as previously described (Tada et al. 1986). Briefly, cells were seeded at a density of 3×10^4 cells/well in a 48-well plate (Corning, NY, USA), allowed to adhere overnight, and culture medium was replaced with fresh serum-free DMEM. MTT was freshly prepared at 0.5 mg/ml in phosphate-buffered saline (PBS). After exposing cells to MHY498 at various concentrations (0.5–32 μM) for 24 h, 500 μL aliquots of MTT stock solution were added to each well, and the plate was incubated at 37 °C for 2 h in a humidified 5 % CO_2 incubator. After 2 h, the medium was removed and 500 μL of ethanol–DMSO (1:1 mixture solution) was added to each well to dissolve the formazan. After 10 min, the optical density of each well was read at 560 nm using a spectrophotometer. The results are the means of six experiments.

Assay of mushroom tyrosinase inhibition

Mushroom tyrosinase was used throughout the study. Tyrosinase activity was determined as described previously with minor modification (No et al. 1999). Briefly, 20 μL of an aqueous solution of mushroom tyrosinase (1,000 units) was added to each well of a 96-well microplate (Nunc, Denmark), in a total assay volume of 200 μL containing a 1 mM L-tyrosine solution, and 50 mM phosphate buffer (pH 6.5). Assay mixtures were then incubated at 25 °C for 30 min, and amounts of dopachrome produced were determined spectrophotometrically at 492 nm (OD_{492}) using a microplate reader (Hewlett Packard). Tyrosinase activity is expressed as IC_{50}, that is, the concentration that caused 50 % inhibition, which was read off the *X*-axis of the dose–response curve. In the present study, dose-dependent inhibition experiments were performed in triplicate to determine the IC_{50} values of MHY498 and kojic acid. Log–linear curves and their equations were determined by averaging inhibitions at three doses, 1, 5, and 10 μM of MHY498 and 20, 30, and 40 μM of Kojic acid.

Kinetics of tyrosinase inhibition

Various concentrations of L-DOPA (0.5–4 mM) substrate, 20 μL of an aqueous mushroom tyrosinase solution (1,000 units), and 50 mM of potassium phosphate buffer (pH 6.5) with or without a test sample (0, 5, 10 μM MHY498) were added to a 96-well plate into a total volume of 200 μL. Initial rates of DOPAchrome formation were determined by measuring increases in absorbance at 492 nm per min (ΔOD_{492}/min) using a microplate reader. The type of inhibition of the compounds was determined by a modification of the Michaelis–Menten equation and Lineweaver-Burk plots at varying L-DOPA concentrations.

Tyrosinase activity assay in B16F10 melanoma cells

Tyrosinase activity was determined in B16F10 melanoma cells by measuring the rate of L-DOPA oxidation. Cells were seeded into the wells of a 24-well plate at a density of 5×10^4 cells/well, incubated in the presence or absence of 100 nM α-MSH, and then treated for 48 h with various concentrations of MHY498 (0.5–16 μM) or 16 μM of kojic acid. After washing, cells were lysed in 100 μL of 50 mM sodium phosphate buffer (pH 6.5) containing 1 % Triton X-100 (Sigma) and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), and frozen at −80 °C for 30 min. After thawing, cellular extracts were clarified by centrifugation at 12,000 g for 30 min at 4 °C, and 80 μL of the supernatants obtained and 20 μL of L-DOPA (2 mg/mL) were placed into the wells of a 96-well plate. Absorbances were read at 492 nm every 10 min for 1 h at 37 °C using an ELISA plate reader. Final activities were expressed as ΔOD/min.

Determination of melanogenesis level in B16F10 melanoma cells

Melanin contents were determined using a modification of the method described by (Bilodeau et al. 2001). In the current study, the amount of melanin was used as an index of melanogenesis. B16F10 melanoma cells (5×10^4 cells/well) were seeded in a 24-well plate and incubated in the presence or absence of 100 nM α-MSH. Cells were then incubated for 48 h with various concentrations of MHY498 (0.5–16 μM) or 16 μM of kojic acid. After washing twice with PBS, cells were detached by incubation in trypsin/EDTA, and dissolved in 100 μL of 1 N NaOH. The samples obtained were then incubated at 60 °C for 1 h and mixed to solubilize the melanin. Melanin contents were determined by measuring absorbances at 405 nm.

Cell lysis and Western blot analysis

Cells were washed with PBS, and then treated with ice-cold PBS. Pellets were harvested at 1,000 × *g* at 4 °C for 5 min. The pellets were suspended in buffer A (10 mM Tris, pH 8.0 with 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 % NP-40, 1 μM pepstatin, and 1 mM *p*-aminobenzamidine), incubated on ice for 15 min, and then centrifuged at 14,000 *g* at 4 °C for 15 min. The supernatants were used as the cytosolic fractions and the

pellets were resuspended in buffer C (10 mM Tris, pH 8.0 with 50 mM KCl, 100 mM NaCl, 1 μM pepstatin, and 1 mM *p*-aminobenzamidine) and incubated on ice for 30 min, then centrifuged at 14,000 *g* at 4 °C for 30 min. The resultant supernatants were used as the nuclear fraction.

Cell lysates (containing 20 μg of protein) were boiled for 5 min in gel-loading buffer (0.125 M Tris–HCl, pH 6.8, 4 % SDS, 10 % 2-mercaptoethanol, and 0.2 % bromophenol blue) at a 1:1 ratio. Total protein equivalents were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10 % acrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and membranes were immediately placed into blocking buffer (5 % non-fat milk) in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1 % Tween 20. Blots were blocked at room temperature for 1 h. Antibody labeling was detected using West-zol Plus and chemiluminescence FluorchemTM SP (Alpha Innotech Co., San Leandro, CA, USA). Pre-stained protein markers were used for molecular weight determinations.

In silico docking of tyrosinase and MHY498

Among many tools available for in silico protein–ligand docking, DOCK6.3 and AutoDock4.2 are the most commonly used due to their automated docking capability. In the present study, we used AutoDock4.2 for the docking simulation of tyrosinase and MHY498. The program performs ligand docking by using a set of predefined 3D grids of the target protein and a systemic search technique (Moustakas et al. 2006; Morris et al. 2009). To prepare for the docking procedure, we performed the following: (1) conversed 2D into 3D structures, (2) calculated charges, and (3) added hydrogen atoms using the ChemOffice program (http://www.cambridgesoft.com).

Statistical analysis

Inhibitions of tyrosinase activities are expressed as percent inhibitions, defined as: $100 - [(A \times 100)/B]$, where $A = OD_{492}$ with a test sample and $B = OD_{492}$ without a test sample. Values are presented as means. The statistical significance of differences between groups was determined by one-factor analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Statistical significance was accepted for p values < 0.05.

Results

The inhibitory effect of MHY498 on mushroom tyrosinase activity

(*Z*)-5-(2,4-Dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498) was tested for its inhibitory effects on mushroom tyrosinase activity using L-tyrosine as a substrate. As shown in Table 1, MHY498 inhibited tyrosinase activity in a dose-dependent manner. In addition, MHY498 (mean $IC_{50} = 3.55$ μM) was significantly more potent than kojic acid, a strong tyrosinase inhibitor (mean $IC_{50} = 22.79$ μM).

The kinetics of MHY498 in the inhibition of mushroom tyrosinase

To explore the mechanism responsible for tyrosinase inhibition by MHY498, we conducted a kinetic study. The mode of enzyme inhibition was determined by Lineweaver-Burk plot analysis as shown in Fig. 3. MHY498 at 5 or 10 μM had K_m values of 2.48 and 5.29 mM, respectively, and the same V_{max} values of 1.74×10^{-2} mM/min (Table 2). As the concentration of MHY498 was increased, the K_m for tyrosinase increased in a dose-dependent manner without changing V_{max}, indicating MHY498 is a competitive inhibitor of tyrosinase.

In silico docking of tyrosinase and binding residues that interact with MHY498

The docking simulation using AutoDock4.2 produced significant scores. The binding energies of MHY498 and kojic acid were −5.83 and −3.91 kcal/mol, respectively. The docking score between a ligand and a receptor is represented by various energy terms, such as, electrostatic energy and van der Waals energy terms, and the solvation energy.

The docking simulation using AutoDock4.2 showed that the binding affinity of MHY498 was greater than that of the kojic acid, a positive control. Using AutoDock4.2, we searched for binding residues within tyrosinase that were close to the inhibitor. As shown in Fig. 4, both MHY498

Table 1 IC_{50} values for tyrosinase inhibition by MHY498 and kojic acid

Compound	Concentration (μM)	Inhibition (%)[a]	IC_{50}(μM)[b] (average)
MHY498	1	45.41 ± 2.21	3.55
	5	53.82 ± 0.5	
	10	58.41 ± 1.32	
Kojic acid	20	46.45 ± 8.58	22.79
	30	58.76 ± 15.77	
	40	66.75 ± 6.62	

Each value is mean ± SE ($n = 3$). Repeated experiments showed the similar results

[a] Tyrosinase inhibition was measured using L-tyrosine as substrate at 1 mM

[b] 50 % Inhibitory concentration (IC_{50})

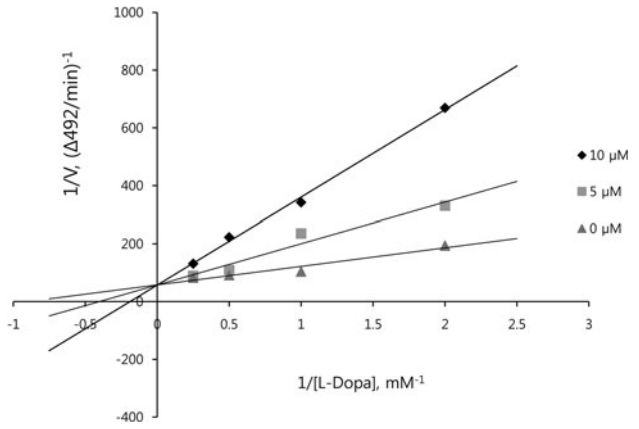

Fig. 3 Lineweaver-Burk plot of mushroom tyrosinase in the presence of MHY498. Results are mean 1/V values of three independent tests performed using different concentrations of L-DOPA substrate

Table 2 Kinetic analysis of MHY498

Concentration (μM)	V_{max} (mM/min)	K_m (mM)
No inhibitor	1.73×10^{-2}	1.11
MIIV498 5.0	1.74×10^{-2}	2.48
MI1Y498 10.0	1.74×10^{-2}	5.29

Each data is mean value of 1/V, the inverse of rate of change of absorbance per minute at a wavelength of 492 nm (ΔA_{492}/min), of three independent tests performed using different concentrations of L-DOPA as a substrate. The Lineweaver-Burk plot's equation is: $1/V = K_m/V_{max} \times 1/[S] + 1/V_{max}$ where V is the reaction velocity (the reaction rate), K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity, [S] is the substrate concentration

and kojic acid were found to hydrogen bond with the MET 280 residue of tyrosinase.

Effect of MHY498 on B16F10 melanoma cell viability

Some well-known tyrosinase inhibitors, such as, hydroquinone and kojic acid, are cytotoxic, and thus, we examined the cytotoxicity of MHY498 using B16F10 melanoma cells using an MTT assay. The results obtained indicated that MHY498 is relatively non-cytotoxic to B16F10 melanoma cells (Fig. 5).

Inhibition of tyrosinase activity in B16F10 melanoma cells by MHY498

To investigate the mechanism whereby MHY498 inhibits melanin biosynthesis, we examined the inhibitory effect of MHY498 on cellular tyrosinase activity in B16F10 melanoma cells treated with α-MSH. As shown in Fig. 6, MHY498 inhibited α-MSH-stimulated tyrosinase activity in these cells. After 48 h of incubation with MHY498, tyrosinase activity was 216.35 % at 0.5 μM, 172.53 % at 4 μM, and 142.33 % at 16 μM, whereas in the control group treated with α-MSH alone it was 248.52 %. At 16 μM, the tyrosinase activities of MHY498 and kojic acid were 142.33 and 199.08 %, respectively, indicating that MHY498 is a more potent inhibitor of tyrosinase activity.

Inhibition of melanin production in B16F10 melanoma cells by MHY498

To assess the inhibitory effect of MHY498 on melanogenesis, we quantified the melanin contents of B16F10 melanoma cells treated with MHY498. The inhibitory effect of MHY498 on melanin content is shown in Fig. 7. Melanin contents in B16F10 melanoma cells treated with MHY498 decreased dose-dependently; percentage melanin contents were 101.53 % at 0.5 μM, 90.47 % at 4 μM, and 78.92 % at 16 μM, as compared with 128.25 % in cells treated with α-MSH alone. These results show that MHY498 inhibits melanogenesis at concentrations that do not significantly affect cell viability. At 16 μM, the percentage melanin content in cells treated with MHY498 or kojic acid were 78.92 and 84.67 %, respectively, indicating that MHY498 is the more potent inhibitor of melanin production.

Effects of MHY498 on the expression of tyrosinase and on the cAMP-dependent signaling pathway

It has been shown that the cyclic AMP (cAMP) pathway plays a pivotal role in α-MSH-induced melanogenesis. Furthermore, it has been shown that the molecular events associated with this pathway involve the activations of protein kinase A (PKA) and cAMP responsive element binding protein (CREB) transcription factor, and that these activations lead to the up-regulation of microphthalmia associated transcription factor (MITF) expression (Busca and Ballotti 2000).

To investigate the molecular events underlying the anti-melanogenic effect of MHY498, we examined the levels of PKA, pCREB (phosphorylated CREB, active form), MITF, and tyrosinase in B16F10 melanoma cells treated with MHY498 (0.5, 4, 16 μM). To determine the activation of PKA, we measured the phosphorylation of regulatory subunit of PKA at Ser 96 [pPKA RIIα (Ser96)] which decreases the affinity of regulatory subunit for catalytic subunit of PKA (Han et al. 2012; Mauban et al. 2009; No et al. 2004; Yang et al. 2011). As shown in Figs. 8 and 9, MHY498 dose-dependently suppressed pPKA RIIα (Ser96), tyrosinase, pCREB, and MITF protein levels, and

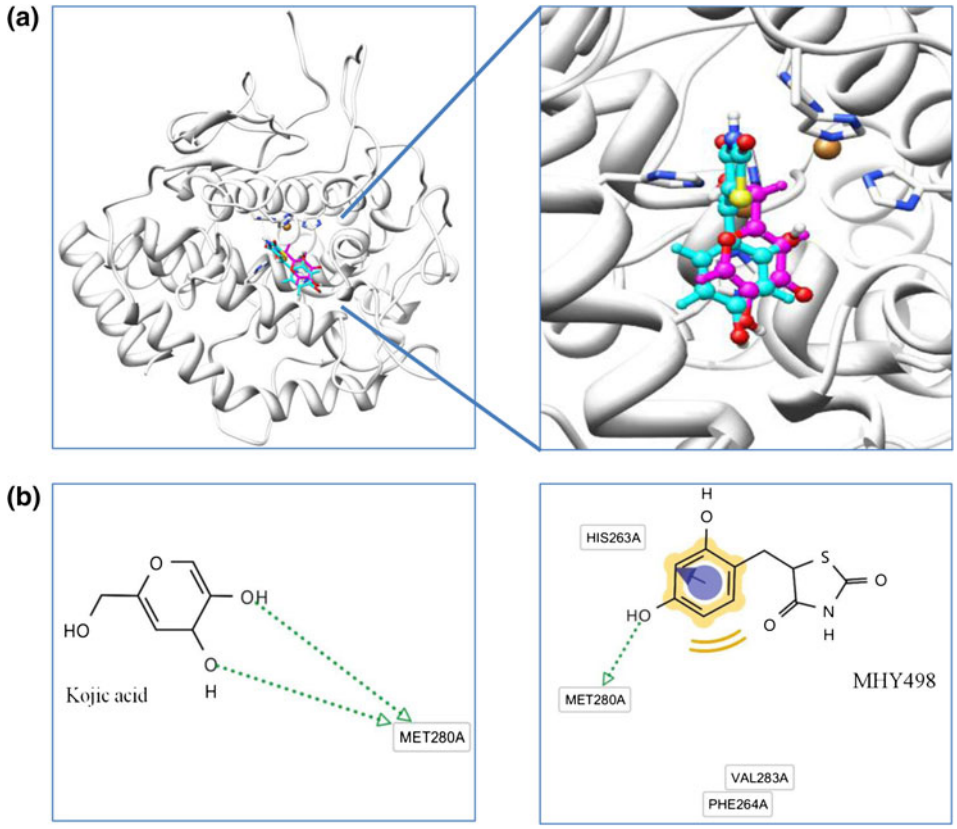

Fig. 4 Computational structure prediction for tyrosinase and docking simulations with MHY498 and kojic acid. (**a**) Docking simulation results between the predicted 3D structure of mushroom tyrosinase and the chemical structures of MHY498 (*cyan* color) and kojic acid (*magenta* color). Docking simulations were performed using AutoDock4.2. The binding energies of MHY498 and kojic acid were −5.83 and −3.91 kcal/mol, respectively. (**b**) The *green lines* represent possible hydrogen bonds. MET 280 of tyrosinase was predicted to hydrogen bond to MHY498 and to kojic acid

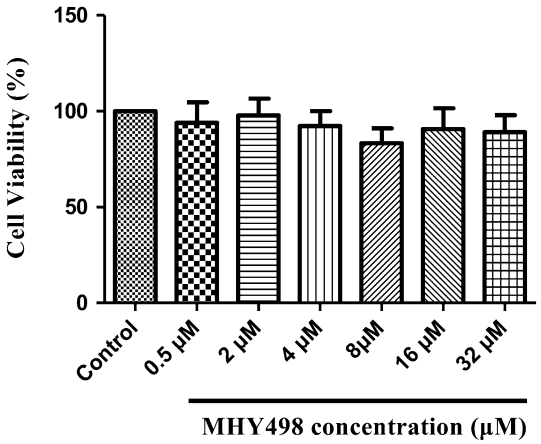

Fig. 5 The effect of MHY498 on the viability of B16F10 melanoma cells. Cells were treated with various concentrations (0.5–32 μM) of MHY498 for 24 h and subjected to an MTT assay. The result is expressed as a percentage of the control, and each data is expressed as mean ± SE ($n = 6$)

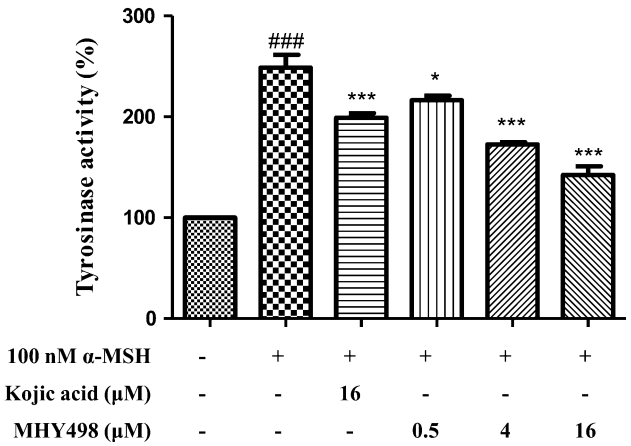

Fig. 6 The inhibitory effect of MHY498 on tyrosinase activity in B16F10 melanoma cells in the presence of 100 nM α-MSH. B16F10 melanoma cells were treated with various doses of MHY498 (0.5–16 μM) for 24 h. The result is expressed as a percentage of control and each data is expressed as mean ± SE ($n = 6$). Statistical results of one-factor ANOVA: $^{###}p < 0.001$ versus the control group, $^{***}p < 0.001$, $^{*}p < 0.05$ versus the group stimulated with only α-MSH, respectively

significant differences have not found in the total PKA and total CREB.

These results implied that reduced tyrosinase levels in B16F10 melanoma cells treated with MHY498 was due to a blockade of the cAMP-dependent signaling pathway involving PKA, CREB, and MITF.

Discussion

Melanin is principally responsible for skin color and plays an important role in the prevention of skin injury under normal physiological conditions, but on the other hand, abnormal

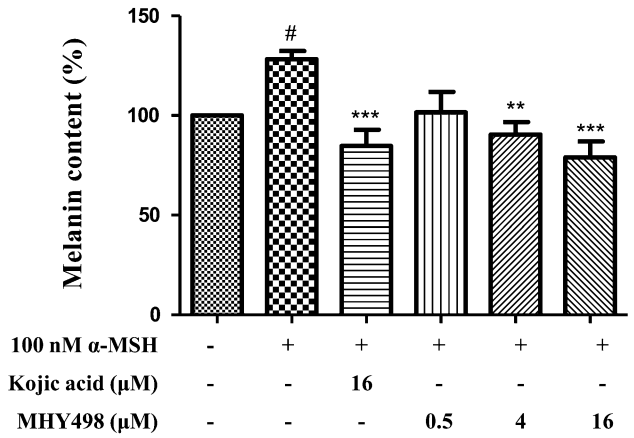

Fig. 7 Effect of MHY498 on melanin contentsin B16F10 melanoma cells in the presence of 100 nM α-MSH. Amounts of melanin were measured as described in methods. The result is expressed as a percentage of control and each data is expressed as mean ± SE ($n = 9$). Statistical results of one-factor ANOVA: $^{\#}p < 0.05$ versus the control group, $^{***}p < 0.001$, $^{**}p < 0.01$ versus the group stimulated with only α-MSH, respectively

pigmentation manifesting as freckles, age spots, or melasma sometimes causes serious problems. Therefore, the modulation of melanogenesis is viewed as an important strategy for the treatment of abnormal skin pigmentation (Jones et al. 2002). Tyrosinase inhibition has been the subject of numerous studies, and several inhibitors are currently used as cosmetic additives and medicinal products for the treatment of hyperpigmentation. In addition, demands for safer and more effective anti-pigmentation products continue to increase.

In the present study, we synthesized (*Z*)-5-(2,4-dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498) (Figs. 1, 2) by Knoevenagel condensation and investigated its tyrosinase inhibitory effects. MHY498 was found to reduce mushroom tyrosinase activity dose-dependently, and our kinetic study showed that MHY498 is a competitive inhibitor of mushroom tyrosinase with the V_{max} value of 1.74 ×

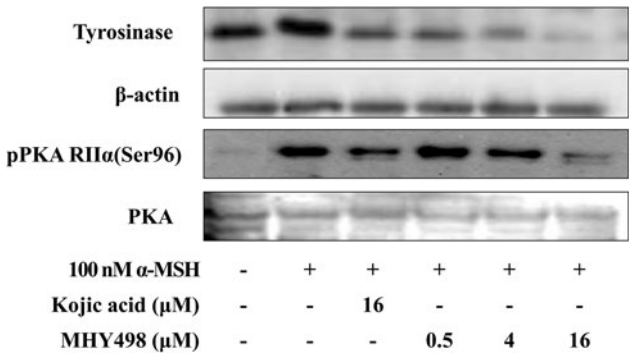

Fig. 8 Effects of MHY498 on the levels of tyrosinase and pPKA RIIα (Ser96). Cytosolic protein levels were measured by Western blotting. Samples were resolved on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes. β-Actin was used as a loading control

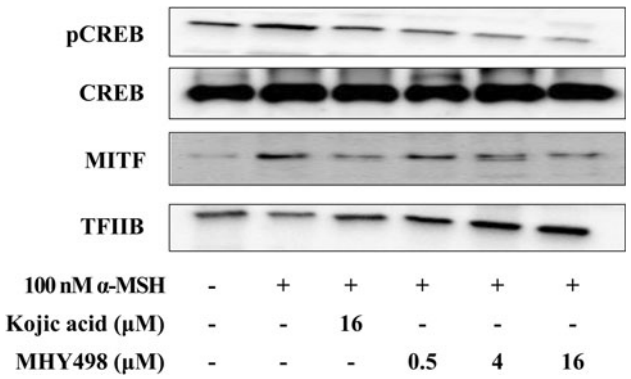

Fig. 9 Effects of MHY498 on the levels of pCREB and MITF. Nuclear protein levels were measured by Western blotting. Samples were resolved on 10 % SDS-PAGE gels and transferred to nitrocellulose membranes. TFIIB was used as a loading control

10^{-2} mM/min (Fig. 3; Table 2). Most inhibitors of melanogenesis, especially inhibitors, such as, tropolone (Fitzpatrick 1971) and kojic acid (Cabanes et al. 1994), chelate copper in the tyrosinase active site, and thus, act as competitive inhibitors. 4,4′-Dihydroxybiphenyl (No et al. 2004), protocatechuic aldehyde (No et al. 2006), and 3,4-dihydroxyacetophenone (Kim et al. 2006) share a hydroxy-phenyl group, which possible means that their competitive tyrosinase inhibitory activities are due to their structural similarities with tyrosinase substrates like L-tyrosine and L-DOPA.

Docking simulation using AutoDock4.2 showed that direct tyrosinase inhibition of MHY498 occurs at the active site. Furthermore, the binding energy (−5.83 kcal/mol) between tyrosinase and MHY498 calculated by the docking program was higher than that (−3.91 kcal/mol) of kojic acid. Although the inhibitory mechanism of MHY498 needs to be confirmed by a future X-ray crystallographic study, this result suggests that MHY498 inhibits tyrosinase more so than kojic acid. According to the docking program, the binding residue interacting with MHY498 or kojic acid was MET 280 of tyrosinase (Fig. 4), which indicates that MHY498 might inhibit tyrosinase activity by binding at the active site of mushroom tyrosinase.

Interestingly, MHY498 was not cytotoxic to B16F10 melanoma cells at the concentrations tested (Fig. 5), and MHY498 (mean $IC_{50} = 3.55$ μM) was a 6.4-fold more potent tyrosinase inhibitor than kojic acid (mean $IC_{50} = 22.79$ μM). Furthermore, it was found in B16F10 melanoma cells, MHY498 dose-dependently inhibited both tyrosinase activity and melanin production (Figs. 6, 7), which suggests that the anti-melanogenic effects of MHY498 are not associated with undesirable cytotoxicity.

In our previous studies, in order to search for potent skin-whitening agents, we designed and synthesized several thiazolidine derivatives, such as, 5-(substituted benzylidene) thiazolidine-2,4-dione derivatives and 2-(substituted

phenyl)thiazolidine-4-carboxylic acid derivatives, and examined their tyrosinase inhibitory effects, kinetics, and effects on melanin content in B16F10 cells. Subsequently, we found that some of these derivatives inhibited mushroom tyrosinase at levels similar to that of kojic acid (Ha et al. 2012a, b). However, as compared with these derivatives, MHY498 has a very low IC_{50} value (3.55 μM).

According to our previous studies and other related studies, tyrosinase gene expression, and cAMP production are enhanced during α-MSH-induced melanogenesis (Busca and Ballotti 2000; Ebanks et al. 2009; Han et al. 2012; Gasperini et al. 2002; Bertolotto et al. 1998). cAMP activates PKA, which increases the expressions of tyrosinase and MITF. The second of these, MITF is a melanocyte-specific transcription factor and plays a pivotal role in melanocyte survival, development, and differentiation, which all occur via the activation of PKA. Activated PKA phosphorylates CREB and this activates the expressions of specific genes containing consensus CRE (cAMP responsive element) sequences (5′-TGACCTCA-3′) in their promoters (Busca and Ballotti 2000).

To determine the intracellular mechanism responsible for the effects of MHY498, we attempted to identify those cellular factors involved in melanogenesis that are expressionally affected by MHY498. α-MSH increased the protein levels of pPKA RIIα(Ser96), pCREB and MITF (as determined by Western blotting), and MHY498 effectively and dose-dependently reduced these levels (Figs. 8, 9), and led to the suppression of tyrosinase expression (Fig. 8). These findings suggest that the suppression of α-MSH-stimulated melanogenesis by MHY498 is mediated by blockade of the cAMP/PKA/CREB pathway and reduced MITF expression.

In conclusion, MHY498 was found to be a potent tyrosinase inhibitor that suppresses tyrosinase activity and total melanin contents in B16F10 melanoma cells without adversely affecting cell viability. These findings suggest that MHY498 could be a useful antimelanogenic agent for the prevention and treatment of disorders related to skin pigmentation. Furthermore, our molecular studies indicate that the anti-melanogenic effect of MHY498 is probably due to the direct inhibition of tyrosinase activity and the inhibition of gene expressions of tyrosinase and MITF via the modulation of the cAMP/PKA/CREB pathway.

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