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Characterization of a novel tyrosinase inhibitor, (2*RS*,4*R*)-2-(2,4-dihydroxyphenyl) thiazolidine-4-carboxylic acid (MHY384)

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ABSTRACT

Background: We synthesized (2*RS*,4*R*)-2-(2,4-dihydroxyphenyl)thiazolidine-4-carboxylic acid (MHY384) as a potential tyrosinase inhibitor and investigated its antityrosinase activity.
Methods: The structure of MHY384 was established using ^{1}H and ^{13}C NMR spectroscopy and mass spectral analyses. To investigate dual mechanisms of action of MHY384 for the inhibition of melanin synthesis, we confirmed the inhibitory effect of tyrosinase catalytic activity of MHY384. Then, we confirmed the inhibitory effect of MHY384 on transcription of tyrosinase mRNA through alpha-MSH-induced cAMP–PKA–MITF signaling. In addition, we supported the inhibitory mechanism of MHY384 against tyrosinase using a kinetic study and docking programs.
Results: To determine how MHY384 regulates melanogenesis, we measured melanin levels and expression of the genes for microphthalmia-associated transcription factor (MITF) and tyrosinase in α-melanocyte-stimulating hormone (α-MSH)-induced B16F10 melanoma cells. MHY384 potently inhibited tyrosinase activity and melanin production in B16F10 melanoma cells. Through docking models, we were able to construct the tertiary structure of mushroom tyrosinase and simulate its docking with MHY384. The result supports that MHY384 strongly interacts with tyrosinase residues in the active site and it can directly inhibit tyrosinase. To investigate additional mechanisms of action of MHY384, we confirmed that the inhibition of tyrosinase activity was found to be due to the modulation of the expression of tyrosinase and its transcription factor, MITF, through cAMP, which regulates protein kinase A.
Conclusions: This study strongly indicates that the depigmenting effect of MHY384 results from the down-regulation of MITF and tyrosinase through direct tyrosinase inhibition.
General significance: Our findings suggest that MHY384 can be an effective skin-whitening agent.

1. Introduction

Melanogenesis is the process by which melanin is secreted by melanocytes located in the basal layer of the dermis. Melanin is the major cellular component responsible for skin color and is the source element of pigmentation that appears as spots and freckles on the skin [1]. Although melanogenesis is a necessary defense mechanism against UV irradiation and excess oxidative stress, abnormal accumulation of melanin pigment can cause hyperpigmentation, including melasma, freckles, and senile lentigines [2,3]. Therefore, a number of whitening compounds have been screened for their effectiveness in reducing melanogenesis. Consequently, these tyrosinase regulators may not only control melanin production but also lead to the development of new therapies for pigmentation or depigmentation.

The production of melanin by the melanocytes in skin is controlled by α-melanocyte-stimulating hormone (α-MSH), a physiological ligand that binds to melanocortin-1 receptors (MC1R) [4]. Stimulation

Abbreviations: ANOVA, Analysis of variance; CREB, cAMP responsive element binding protein; CO_2, Carbon dioxide; CBP, CREB-binding protein; cAMP, Cyclic adenosine monophosphate; $CDCl_3$, Deuterated chloroform; L-DOPA, L-3,4-dihydroxyphenylalanine; DMSO, Dimethyl sulfoxide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; HCl, Hydrogen chloride; NMR, Nuclear magnetic resonance; MC1R, Melanocortin-1 receptors; α-MSH, α-Melanocyte-stimulating hormone; MITF, Microphthalmia-associated transcription factor; PMSF, Phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene difluoride; PBS, Phosphate-buffered saline; PDB, Protein data bank; PKA, Protein kinase A; NaCl, Sodium chloride; NaOH, Sodium hydroxide; UV, Ultraviolet

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of MC1R by α-MSH results in the activation of cyclic adenosine monophosphate (cAMP). Activation of cAMP leads to the phosphorylation of protein kinase A (PKA), which induces the expression of microphthalmia-associated transcription factor (MITF). MITF, in turn, plays a key role in the expression of the tyrosinase gene [5,6].

Tyrosinase is a copper-containing enzyme that is widespread in nature and is mainly involved in pigmentation [7]. This key enzyme catalyzes the monophenolase hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA), and then, the oxidation of L-DOPA to DOPA quinone. The latter is a substrate for the synthesis of pheomelanins or eumelanins, which are red-yellow or black-brown pigments [8]. Therefore, the regulation of tyrosinase has been useful for the treatment of pigmentation disorders and in the development of cosmetic whitening agents.

Various tyrosinase inhibitors have been found in mammalian melanocytes and melanoma cells that regulate melanin synthesis by modulating tyrosinase activity *in vitro* and *in vivo*, and they have been classified by their mechanisms of action [9,10]. One mechanism is inactivation of the enzyme, and the other is competitive displacement of the tyrosinase substrates, L-tyrosine and L-DOPA, with other chemically related dihydroxybenzene derivatives. Purified mushroom tyrosinase have been used in most studies that have attempted to identify melanogenesis inhibitors. There are many tyrosinase inhibitors such as hydroquinone [11], ascorbic acid derivatives [12], azeleic acid [13], retinoids [14], arbutin [15], and kojic acid [16]. However, some well-known whitening agents, such as hydroquinone and kojic acid, are considered as harmful agents because of their undesirable side effects such as cytotoxicity, skin cancer, and dermatitis. Therefore, safe and effective whitening agents are needed.

Recently, our laboratory reported a new family of hydroxy-substituted phenyl naphthalene analogs of resveratrol; these analogs inhibited tyrosinase activity and reduced melanin level in B16F10 melanoma cells [17,18]. In addition, compounds that inhibit other melanogenesis signaling proteins have been discovered, including linoleic acid and terrain, which inhibit melanogenesis by downregulating tyrosinase expression [19,20].

These reports led us to search for new tyrosinase inhibitors. We synthesized a series of L-tyrosine derivatives in order to identify novel tyrosinase inhibitors. One such derivative, MHY384, showed strong tyrosinase-inhibitory activity and was selected for further study. Here, we report the characterization and evaluation of MHY384, an L-tyrosine derivative. We investigated the inhibitory effects of MHY384 on tyrosinase activity and the regulatory effects on melanogenesis in B16F10 melanoma cells. The analysis was performed by using a mushroom tyrosinase and quantifying the expression of tyrosinase and MITF, cAMP levels, and phosphorylation of PKA. In addition, studies on the inhibitory mechanism of MHY384 against tyrosinase were conducted by using a kinetic study and docking programs.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase (>60% pure according to SDS-PAGE analyses), L-tyrosine, kojic acid, α-MSH, and other chemical reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies against MITF, tyrosinase, pPKA, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA). Assay kits for cAMP were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Cell culture system

B16F10 cells (from the Korean Cell Line Bank) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical) with 10% fetal bovine serum (FBS; Sigma Chemical) and penicillin/streptomycin (100 IU/50 μg/ml) in a humidified atmosphere containing 5% CO_2 (in air) at 37 °C. B16F10 cells were cultured in 24-well plates for melanin quantification and enzyme activity assays.

2.3. Cell viability

Cell survival was quantified by following the method of Tada et al. [21] and using a colorimetric MTT assay that measures mitochondrial activity in viable cells. This method is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical) to MTT-formazan crystal by a mitochondrial enzyme as previously described. Briefly, cells seeded at a density of 3×10^4 cells/well in a 48-well plate (Corning, NY, USA), were allowed to adhere overnight; the culture medium was then replaced with fresh serum-free DMEM. MTT was freshly prepared at 0.5 mg/ml in phosphate-buffered saline (PBS). After cells were exposed to MHY384 at various concentrations for 24 h and 48 h, respectively, 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 EtOH–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 by a spectrophotometer. The optical density (OD) of the formazan formed in the control cell wells was set at 100% in the enzymatic assay for tyrosinase activity.

2.4. Assay to measure inhibition of mushroom tyrosinase

Mushroom tyrosinase was used as the source of the enzyme for the entire study. Tyrosinase activity was determined as described previously with minor modification [22]. Briefly, 20 μl of an aqueous solution of mushroom tyrosinase (1000 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). The assay mixture was incubated at 25 °C for 30 min. After incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm (OD_{492}) by using a microplate reader (Hewlett Packard). Tyrosinase activity was calculated as the IC_{50}, the concentration of drug that causes 50% inhibition, which was obtained from 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} of each drug. The log-linear curves and their equations were determined by the average inhibition of the 3 doses. The IC_{50} value was calculated as the concentration of inhibitor (on the X-axis) at 50% inhibition (on the Y-axis).

2.5. Kinetic analysis of tyrosinase inhibition

Various concentrations of L-tyrosine (1 to 8 mM) substrate, 20 μl of an aqueous mushroom tyrosinase solution (1000 units), and 50 mM potassium phosphate buffer (pH 6.5) with or without a test sample [0.5 to 2.0 μM MHY384] were added to a 96-well plate in an assay mixture with a total volume of 200 μl. The initial rate of DOPA chrome formation in the reaction mixture was determined by the increase in absorbance at 492 nm per min (ΔOD_{492}/min) read by a microplate reader. The Michaelis constant (K_m) and maximal velocity (V_{max}) of tyrosinase activity were determined by using Lineweaver–Burk plots with various concentrations of L-tyrosine substrate. The reaction kinetics required a modification of the Michaelis–Menten equation due to competitive inhibition by MHY384 and substrate inhibition by L-tyrosine.