A novel anti-melanogenic agent, KDZ-001, inhibits tyrosinase enzymatic activity

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\textbf{ABSTRACT}

\textit{Background:} The demand for anti-melanogenic agents is increasing due to the unwanted side effects of current treatments. To find an effective anti-melanogenic agent, we used zebrafish as a whole animal model for phenotype-based drug and cosmetic discovery screening.

\textit{Objectives:} The aim of this study was to identify and explore a small molecule that could be used for skin-whitening cosmetics.

\textit{Methods:} Using zebrafish embryos, we examined the effects of 1000 compounds on zebrafish development and pigmentation. Pigmentation production was assessed by tyrosinase (TYR) enzymatic activity and melanin contents. Pigmentation marker expression in the human melanoma cell line HMV-II was analyzed by western blot. We also tested reconstituted human skin tissue and analyzed KDZ-001 with computational molecular modeling.

\textit{Results:} We identified three compounds that affected the pigmentation of developing melanophores in zebrafish. Among them, we identified KDZ-001, a novel anti-melanogenic agent, which strongly inhibits melanin synthesis in the developing melanophores of zebrafish, HMV-II cells, and reconstituted human skin with no toxicity. We found that KDZ-001 directly inhibits TYR enzymatic activity. Notably, computational molecular modeling of KDZ-001 suggested that its interaction with copper ions in the active site of TYR is essential for melanin synthesis, further demonstrating that KDZ-001 mainly acts as a TYR inhibitor to synthesize melanin.

\textit{Conclusion:} KDZ-001 inhibits melanin synthesis and has a potential for use in skin-whitening cosmetics.

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1. Introduction

Skin primarily consists of three different cell types: keratinocytes, fibroblasts, and melanocytes. Melanocytes are specialized cells that synthesize pigment, which is also known as melanin. Melanin is a heterogeneous biopolymer that is produced in melanosomes, which are organelles that synthesize, store, and transport melanin through a series of enzymatic reactions known as melanogenesis. Melanogenesis involves the catalysis of tyrosine by TYR-family proteins, including TYR and TYR-related protein 1/2 (TRP-1/2) \cite{1,2}.

Hyper-pigmented, dark-skinned individuals are more resistant to skin damage caused by ultraviolet (UV) radiation than light-colored individuals \cite{3}. The large amount of melanin in the epidermis of dark skin protects against UV radiation by acting as a neutral density filter \cite{4}. Moreover, melanin neutralizes reactive oxygen species via its antioxidant and free radical-scavenging activities \cite{5,6}. Despite the positive effects of melanin synthesis in the skin, a particular condition known as melanoma involves hyperpigmentation of the...
skin and requires control of skin pigmentation using cosmetic skin-whitening agents to inhibit the activity of melanogenesis-related enzymes [7]. Although many anti-melanogenic agents, such as kojic acid, arbutin, and various herbal extracts, are currently available, the demand for anti-melanogenic agents is increasing due to their unwanted side effects.

Microphthalmia-associated transcription factor (MITF) is a transcription factor essential for melanocyte development [8]. The role of MITF in melanocyte differentiation is mediated by activation of the cyclic 5',3'-adenosine monophosphate (cAMP) pathway through the melanocortin signaling pathway, mediating the activation of α-melanocyte-stimulating hormone (α-MSH) [9]. Briefly, activation of α-MSH signaling increases the cytoplasmic cAMP level, which increases transcription of MITF. TYR encodes genes, including those for TRP-1/2, are transcribed by MITF through a highly conserved 10 base pair motif (GTCAATGCT) termed the M-box [10,11]. This key enzyme catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and subsequently catalyzes oxidation of DOPA to DOPAquinone. TYR also catalyzes DOPAchrome to 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid. Finally, these products can be classified as eumelanin (black/brown) and pheomelanin (red/yellow), which are responsible for human skin color in melanocytes [2,12]. Unlike mammals, zebrafish (Danio rerio), a small teleost fish, possesses three types of pigment cells: melanophores (black), xanthophores (yellow), and iridophores (containing reflecting platelets, blue). However, the melanogenesis-related genes in zebrafish, such as TYR, are similar to the mammalian genes [13,14]. For example, the MITF-homolog nacre/mitf regulates the differentiation of melanophores from neural crest cells in zebrafish [15]. Phylogenetic and spatiotemporal expression pattern analyses of tyr in the developing melanophores of zebrafish have been reported [14]. Pigment formation is initiated in the developing retinal pigment epithelium in zebrafish following tyr transcription at 16.5 h post fertilization (hpf), and melanin in melanophores can be visualized in the dorsolateral skin and retina at approximately 24 hpf [14]. Because melanin in the melanophores is synthesized at early stages in zebrafish embryos, melanophores can be visualized under a microscope [16].

The zebrafish is commonly used as an animal model for phenotype-based drug and cosmetic discovery [17]. We have reported that the zebrafish can facilitate a screen of anti-melanogenic agents in vivo and is an alternative to mammalian models [16,18]. In this study, the use of zebrafish for phenotype-based screening allowed us to screen 1000 compounds in the Korea Chemical Bank to identify novel anti-melanogenic agents. In addition to the zebrafish model, we tested HMV-II cells and reconstituted human skin tissue, and used computational molecular modeling. To this end, we identified KDZ-001 as an anti-melanogenic agent and found that it potently inhibited TYR enzymatic activity.

2. Materials and methods

2.1. Maintenance of zebrafish

Zebrafish were maintained under standard conditions as previously described [19]. All experimental protocols involving zebrafish were approved by the Animal Care and Use Committee of the Korea Research Institute of Chemical Technology.

2.2. Phenotype-based screening for anti-melanogenic effects using zebrafish embryos

To screen anti-melanogenic agents, 10 hpf embryos were arranged in a 24-well plate, with 10 embryos per well, containing 1 ml of embryo medium. Test compounds were dissolved in dimethylsulfoxide (DMSO), added to each well, and phenotype-based evaluations of anti-melanogenic effects were performed at 34 hpf. N-phenylthiourea (Sigma-Aldrich, St. Louis, MO, USA) and arbutin (Sigma-Aldrich) were used as positive controls. To acquire images, embryos were de-chloronated using forceps, anesthetized in tricaine (Sigma-Aldrich), and mounted in 3% methylcellulose. Images were acquired using the Leica MZ10 F stereomicroscope, Leica DFC425 camera, and Leica Application Suite software (Ver. 4.5).

2.3. Quantitative measurement of Tyr activity and melanin content in zebrafish embryos

Measurements of Tyr activity and melanin contents were determined as previously described [16]. Briefly, approximately 100 embryos were treated with melanogenic modulators 10–34 hpf or 2.5–3 days post fertilization (dpf), and lysed in Celllytic™M solution (Sigma-Aldrich). The lysate was separated by centrifugation at 12,000 rpm for 5 min. After centrifugation, the aqueous phase was transferred to a new tube, and the protein was quantified using a microplate reader (M1000pro; Tecan, Manne- dorf, Switzerland). A total of 250 μg of protein in 100 μl of aqueous phase was transferred into a 96-well plate, and 100 μl of 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA) was added. The microplate was incubated at 37 °C for 1 h and absorbance was measured at 475 nm. The pellet was dissolved in 1 ml of 1 N NaOH at 100 °C for 30 min, then vigorously vortexed to solubilize the melanin. The sample was transferred to a 96-well plate and absorbance was measured at 490 nm. The results were calibrated with a standard curve of known concentrations of synthetic melanin. Tyr activity and melanin contents were calculated as a percentage of the DMSO control. 1-Phenyl 2-thiourea (PTU) and arbutin were used as a positive control. All experiments were performed at least five times.

2.4. Cell cultures

The human melanoma cell line HMV-II was cultured with Dulbecco’s modified Eagle’s medium/F-12 HAM supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), and 100 units/ml penicillin-streptomycin solution (HyClone). Cultures were maintained at 37 °C with 95% humidity and 5% CO2. Culture media were changed every 48 h, and the cells were sub-cultured once a week.

2.5. Culture of reconstituted human skin tissue

Neoderm-ME (Tego Science, Seoul, Korea), a reconstituted human skin model containing melanocytes, was maintained according to the manufacturer’s instructions. Briefly, Neoderm-ME was removed from the medium-containing agar and transferred onto 12-well plates and incubated at 37 °C in 5% CO2 for 1 day. The Neoderm-ME was treated with each compound for 5 days. Medium was changed every 2 days and the plate was incubated at 37 °C with 95% humidity and 5% CO2. After 5 days, the skin tissues were used for measurement of melanin content and TYR activity.

2.6. Quantitative measurement of TYR activity and melanin content in HMV-II cells and reconstituted human skin tissue

HMV-II human melanoma cells (1 × 10⁴) were seeded in a 100-mm cell culture dish and maintained for 24 h. The cells were treated with each compound for 10 days. The cells were cultured at 37°C with 95% humidity and 5% CO2 and the medium was changed
3 days after treatment. On the last day of treatment, HMV-II cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and detached by trypsin-EDTA for counting with a haemocytometer. Cells ($5 \times 10^6$) were dissolved in 1 N NaOH at 80 °C for 2 h. The resulting cell lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were transferred to a 96-well plate for optical density measurements at 405 nm using a microplate. Neoderm-ME was treated with compounds and maintained as described above. For the melanin assay, Neoderm-ME was washed with DPBS and dissolved in 1 N NaOH at 95 °C for 45 min. The debris was clarified by centrifugation at 10,000 x g for 10 min. The optical density at 405 nm was measured using a microplate reader. For the TYR activity assay, HMV-II cells and Neoderm-ME were lysed with 0.1% Triton X-100 (in DPBS) and CellyticTM M solution, respectively. Lysates were clarified by centrifugation for 5 min at 10,000 x g. After protein determination, lysates containing 2500 μg/100 μl were transferred to a 96-well plate and 50 μl of 10 mM L-DOPA in phosphate-buffered saline (PBS) was added. The mixtures were incubated for 3 h at 37 °C and the absorbance at 475 nm was measured.

2.7. Western blot analysis

HMV-II human melanoma cells ($1 \times 10^6$) were seeded in a 100 mm cell culture dish and maintained for 24 h. The cells were treated with each compound for 5 days. The cells were cultured at 37 °C with 95% humidity and 5% CO2 and the medium was changed at 3 days after treatment. Lysates were prepared by incubation in radioimmunoprecipitation lysis buffer (Thermo Scientific, Waltham, MA, USA) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Proteins (40 μg) were resolved with 4–12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Thermo Scientific) and transferred to polyvinylidene fluoride membranes (Thermo Scientific). Membranes were blocked with 5% nonfat dried milk in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) and incubated with MITF, TYR, TRP-1, and TRP-2 antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) for 3 h. Blots were washed with TBS-T and exposed to horseradish peroxidase-conjugated goat-anti-mouse IgG for 1 h. Bands were detected using SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific) and visualized with the MyECL imager (Thermo Scientific).

2.8. Computational molecular modeling

Homology modeling of zebrafish and human TYR was carried out using the Automated Mode in the SWISS-MODEL [20–22]. The full-length sequences of zebrafish (Q8AYA1) and human (P14679) TYR were acquired from the UniProt database [23]. The crystal structure of Bacillus megaterium Tyr (PDB id: 4D78, chain B) with 35.2 and 35.1% sequence identity with zebrafish and human TYR, respectively, was used as a protein template. Molecular docking was performed using Maestro v11.2 (Schrödinger, Inc., New York, NY, USA) to analyze binding modes of arbutin and KDZ-001 in the homology models of zebrafish and human TYR. The structural defects of homology models were corrected with the Protein Preparation Wizard. The low-energy three-dimensional structures of arbutin and KDZ-001 were generated by LigPrep. Arbutin and KDZ-001 were then docked into the active site of each homology model using the Glide in Extra Precision (XP) mode based on a grid box of 20 x 20 x 20 Å centered on the ligand. The protein–ligand interactions were analyzed by the Discovery Studio Modeling Environment v4.0 (Accelrys Software, Inc., San Diego, CA, USA) and the molecular models of the docked compounds were displayed using PyMOL v1.7.4 (Schrödinger, Inc.).

2.9. Statistical analysis

All data are presented as mean ± standard deviation using GraphPad Prism 6 software (Graphpad Software, Inc., San Diego, CA, USA). An unpaired t-test was used to analyze differences between the two groups, and statistical significance was set at 0.05 and 0.001 (**p ≤ 0.05 and ***p ≤ 0.001).

3. Results

3.1. KDZ-001 treatment impairs melanin synthesis in zebrafish

We report a zebrafish phenotype-based screening model in which developing melanophores inhibit melanin synthesis upon
treatment with melanogenic regulatory compounds [16]. Using this model, we screened 1000 compounds with unknown targets to determine if they could regulate melanin synthesis. After treatment for 24 h (between 10 and 34 hpf), three of these compounds significantly affected pigmentation in the developing melanophores in zebrafish embryos (Fig. S1). Of the three compounds, only KDZ-001 significantly affected melanin synthesis with no developmental defects (Fig. S1B). To evaluate whether KDZ-001 (Fig. 1A) could block melanin synthesis, we compared its anti-melanogenic effect using well-known melanogenic inhibitors, such as arbutin and PTU [16,24], as positive controls (Fig. 1C, D). Indeed, KDZ-001 treatment in zebrafish embryos resulted in anti-melanogenic effects in a dose-dependent manner (Fig. 1E–H) compared to DMSO-treated embryos (Fig. 1B). In addition to the anti-melanogenic effect of KDZ-001, we determined whether it could easily penetrate into the zebrafish embryos. Bioanalysis should be performed to correlate phenotypes with the actual strength of a compound in the animal model. We measured the internal concentration of KDZ-001 in the embryos using mass spectrometry. KDZ-001 treatment increased the concentration in a dose-dependent manner (Fig. S2), indicating that KDZ-001 was present in the embryos and correlated with the phenotype. Therefore, we selected KDZ-001 for further analyses.

Since treatment with 5 μM KDZ-001 showed that pigmented melanophores in the developing zebrafish were present (Fig. 1F), we speculated that KDZ-001 might be inhibited by TYR enzymatic activity because TYR is an oxidase involved in melanin synthesis [25]. To investigate this possibility, we examined enzymatic activity of TYR as well as melanin content in whole embryos followed by KDZ-001 treatment (Fig. 1I–K). After KDZ-001 treatment, TYR enzymatic activity and melanin content were greatly decreased (Fig. 1J, K). These data suggest a critical role for KDZ-001 in melanin synthesis processes by inhibiting TYR enzymatic activity.

3.2. KDZ-001 treatment blocks melanin synthesis in HMV-II cells

The finding that TYR enzymatic activity was reduced in the developing KDZ-001-treated zebrafish melanophores suggests a role in the regulation of TYR enzymatic activity. Thus, we further investigated the effect of KDZ-001 on TYR enzymatic activity in the HMV-II cell line, in which black colored melanin is produced [26]. We examined the color in the pellets after KDZ-001 treatment, in which the melanin changed from black to brown (Fig. 2A). We examined the protein levels of various pigmentation markers, including MITF, TYR, tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase (DCT/TRP-2). Protein levels of TRP-1 were markedly decreased in HMV-II cells, whereas the levels of MITF, TYR, and TRP-2 were not significantly changed (Fig. 2B). Furthermore, when HMV-II cells were treated with KDZ-001, the TYR enzymatic activity and melanin contents also decreased in a dose-dependent manner (Fig. 2C, D), further supporting the notion that KDZ-001 inhibits pigmentation in HMV-II cells as well as zebrafish embryos.

3.3. KDZ-001 treatment attenuates newly-synthesized melanin in pigmented cells in vivo and in vitro

The critical role of KDZ-001 in the production of melanin in zebrafish, as well as HMV-II cells, prompted us to investigate its role in pigmented cells. We selected two distinct models that possess pigmented cells: (1) after 1 dpf the developing zebrafish embryos showed that melanophore differentiation from the neural crest and pigment production in the differentiated melanophores initially occurs at the same time; however, melanophore differentiation from the neural crest is complete at 2 dpf and only the function of pigment production in the differentiated melanophores remained [27]; and (2) the safety and efficacy of whitening, UV protection, and hydration of Neoderm-ME with melanocytes of reconstituted human skin can be tested [28]. By applying these two

![Fig. 2. KDZ-001 treatment blocks melanin synthesis in the human melanoma cell line HMV-II. (A) Melanin pellets from cell lysates. (B) Western blotting using antibodies against MITF, TYR, TRP-1, and TRP-2. GAPDH was used as an endogenous control. Numbers under each panel indicate calculations of band intensity compared to the DMSO control. The percentage of (C) TYR activity and (D) total melanin content compared with the DMSO control.](image-url)
models (Fig. 3A, E) and subsequent validation with TYR enzymatic activity (Fig. 3C, F) and melanin content (Fig. 3D, G) following KDZ-001 treatment, we found that pigment production was decreased compared with arbutin-treated controls (Fig. 3). These data indicated that newly synthesized melanin is attenuated in fully pigmented cells following KDZ-001 treatment.

3.4. KDZ-001 binds copper ions in the active site for TYR enzymatic activity

Because KDZ-001 was implicated in TYR enzymatic activity in pigment production (Fig. 3C, F), we performed homology modeling and molecular docking simulation to analyze the inhibitory effects of arbutin and KDZ-001 for TYR at the atomic level. TYR is a copper-containing enzyme with a binuclear, type 3 copper center within its active sites [29]. Moreover, copper atoms are coordinated with histidine residues [29]. To further investigate the action of KDZ-001, we compared the sequence identity of TYR between zebrafish and human, which showed high sequence identity (60.6%), suggesting that zebrafish and human TYR have a similar protein structure (Fig. S3) and ligand–protein interaction (Fig. S4). The binding modes of arbutin and KDZ-001 to the homology model of human TYR and key interactions revealed by the docking studies are shown in Fig. 4. The hydroxyl group on the benzene ring of arbutin and the allyl group of KDZ-001 make close contact with the copper ions. In the case of arbutin, a hydroxyl group on the glucose

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**Fig. 3.** KDZ-001 treatment attenuates newly synthesized melanin in pigmented cells in vivo and in vitro. (A) Schematic representation of the experimental schedule. (B) Melanin pellets from zebrafish larva lysates. Percentage of (C) TYR activity and (D) total melanin content compared with the DMSO control in the zebrafish larvae. (E) Representative images of cultured human skin tissue (top and middle) and melanin pellets (bottom). Percentages of (F) TYR activity and (G) melanin contents in the human skin tissue.

**Fig. 4.** KDZ-001 binds copper ions in the active site for human TYR enzymatic activity. (A) Arbutin and (B) KDZ-001. The inhibitors and homology model are represented by sticks (green) and ribbon (blue), respectively. The key interactions are drawn as dashed red lines. The key residues and copper ions are represented by sticks (orange) and spheres (salmon), respectively. Histidine residues around copper ions are represented by white sticks.
ring forms a hydrogen bond with Ser375 and the benzene ring shows $\sigma-\pi$ interaction with Val377. The core scaffold of KDZ-001 shows $\pi-\pi$ interactions with Phe347 as well as $\sigma-\pi$ interactions with Val377. Also, the allyl group of KDZ-001 allows more $\pi$ interactions with histidine residues around the copper ions, demonstrating that these more stable hydrophobic interactions of KDZ-001 result in a stronger inhibitory effect on TYR.

4. Discussion

In this study, we screened 1000 compounds and determined that KDZ-001 inhibits TYR enzymatic activity in zebrafish without inducing developmental defects in in vitro cell and tissue culture systems. KDZ-001 treatment resulted in depigmentation and/or skin lightening in embryos mainly via temporal inhibition of melanin synthesis. This depigmentation and/or skin lightening occurred not only in the developing zebrafish melanophores, but also in the fully developed melanocytes. Furthermore, using computational molecular modeling of KDZ-001 at the atomic level, we found that KDZ-001 mainly acts as a TYR inhibitor to synthesize melanin.

Given the advantages of zebrafish embryos/larvae and their growth in water, zebrafish embryos/larvae have been screened using small molecule compounds [17]; however, the actual concentration has not been defined in treated embryos/larvae. In this study, we measured the internal concentration using mass spectrometry after KDZ-001 treatment (Fig. S2). This procedure shows the correlation between any phenotype and the actual strength of a compound in the animal model. In addition to determining the internal concentration of KDZ-001, we investigated developing melanophores and fully pigmented melanophores. In KDZ-001-treated developing zebrafish embryos, most melanins were not synthesized (Fig. 1G, H). However, depigmentation in the zebrafish embryos at 3 dpf was not observed (data not shown). Although visible depigmentation was not observed in fully pigmented cells, KDZ-001 indeed inhibits TYR enzymatic activity and melanin contents (Fig. 3C, D). This phenomenon was further confirmed in the reconstituted human skin (Fig. 3E–G), suggesting that KDZ-001 attenuates the synthesis of new melanin by inhibiting TYR enzymatic activity.

Moreover, a docking model demonstrated that KDZ-001 makes close contact with the copper ions in the active site of TYR, implying that copper chelation by the allyl group of KDZ-001 is responsible for inhibition of TYR activity. This is supported by the finding that KDZ-001 is a strong competitive inhibitor of TYR (Figs. 3, S4). Therefore, the allyl group of KDZ-001 interacts with copper ions, and this interaction contributes to its TYR inhibition activity.

In this study, we showed that KDZ-001 treatment decreases the TRP-1 protein level (Fig. 2B). Thus, we speculated that there are two distinct possibilities for the reduction in the production of pigmentation following KDZ-001 treatment: 1) TRP-1 is encoded by Tyrosinase-related protein 1 (Typr1), a melanocyte-specific gene involved in eumelanin synthesis. Its enzymatic function is important for the production of eumelanin during the melanin synthesis processes [30]; and 2) TRP-1 plays a role in TYR stabilization: the absence of TRP-1 accelerates degradation of TYR and the reduced stability of TYR is partially rescued by exogenous expression of Typr1 [31,32]. Given that the TYR protein level was not significantly changed (Fig. 2B), we can rule out the possibility that TRP-1 accelerates the degradation of TYR. The reduction in TRP-1 protein levels followed by KDZ-001 treatment likely affects eumelanin synthesis because TYR enzymatic activity and melanin content are significantly decreased (Figs. 1–3). Although KDZ-001 treatment was shown to reduce eumelanin synthesis, future analysis should consider how TRP-1 acts to decrease eumelanin synthesis following treatment with KDZ-001.

In summary, we found that KDZ-001, a novel anti-melanogenic agent, inhibits melanin synthesis. KDZ-001 inhibits TYR enzymatic activity, and likely modulates translation of TRP-1. In conclusion, our findings suggest that KDZ-001 is a potential skin-whitening agent for the cosmetic industry.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jdermsci.2017.11.004.

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